Aus der Kinderchirurgischen Klinik und Poliklinik

im Dr. von Haunerschen Kinderspital

der Ludwig-Maximilians-Universität München

Direktor: Prof. Dr. med. Dietrich von Schweinitz

# Genetic and epigenetic aspects of hepatoblastoma development and treatment

Dissertation

zum Erwerb des Doktorgrades der Medizin

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

vorgelegt von

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aus München

2018

Mit Genehmigung der Medizinischen Fakultät

der Universität München

| | |
|---|---|
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| Tag der mündlichen Prüfung: | 28.06.2018 |

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# Abbreviations

| | | |
|---|---|---|
| AFP | - | Alpha feto protein |
| APC | - | Adenomatous polyposis coli |
| AXIN1 | - | Axis inhibition protein 1 |
| AXIN2 | - | Axis inhibition protein 2 |
| BWS | - | Beckwith–Wiedemann Syndrome |
| CHIC | - | Children's Hepatic tumors International Collaboration |
| COG | - | Children's Oncology Group |
| CT | - | Computed tomography |
| CTNNB1 | - | Beta-Catenin |
| CUL3 | - | Cullin 3 |
| FAP | - | Familial adenomatous polyposis coli |
| GPOH | - | Gesellschaft für Pädiatrische Onkologie und Hämatologie |
| HB | - | Hepatoblastoma |
| HCC | - | Hepatocellular carcinoma |
| HDAC | - | Histone deacetylase |
| HDACi | - | Histone deacetylase inhibition |
| HHIP | - | Hedgehog-interacting protein |
| IGFBP3 | - | Insulin-like growth factor binding protein 3 |
| JPTL | - | Japanese Study Group for Pediatric Liver Tumors |
| KEAP1 | - | Kelch Like ECH Associated Protein 1 |
| MRI | - | Magnetic resonance imaging |
| MSH6 | - | MutS Homolog 6 |
| NFE2L2 | - | Nuclear factor (erythroid-derived 2)-like 2 |
| NQO1 | - | NAD(P)H Quinone Dehydrogenase 1 |
| PRETEXT | - | Pretreatment extension of disease |
| RAD17 | - | Cell Cycle Checkpoint Protein RAD17 |
| SAHA | - | Suberoylanilide hydroxamic acid |
| SFRP1 | - | Secreted frizzled-related protein 1 |
| SIOPEL | - | Société Internationale d'Oncologie Pédiatrique – Epithelial Liver |
| TERT | - | Telomerase reverse-transcriptase |
| TLCT | - | Transitional liver cell tumor |
| TP53 | - | Tumor Protein P53 |

# 1. Publications

Eichenmuller M., Trippel F., Kreuder M., **Beck A.**, Schwarzmayr T., Häberle B., Cairo S., Leuschner I., von Schweinitz D., Strom T. M., Kappler R. *The genomic landscape of hepatoblastoma and their progenies with HCC-like features.* Journal of hepatology 2014; 61:1312-20 (Impact (2014)=11.336)

**Beck A.**, Eberherr C., Hagemann M., Cairo S., Häberle B., Vokuhl C., von Schweinitz D., Kappler R. *Connectivity map identifies HDAC inhibition as a treatment option of high-risk hepatoblastoma.* Cancer biology & therapy 2016; 17:1168-76. (Impact (2016)=3.294)

# 2. Introduction

## 2.1 Hepatoblastoma

### 2.1.1 Epidemiology

Hepatoblastoma (HB) is a rare pediatric tumor originating from the liver. Although it is the most common hepatic malignancy in children, it only makes up just over 1% of all pediatric neoplasms[1]. The annual incidence lies somewhere around 1.2-1.5 cases per million children under the age of 15 in Western countries, with almost all cases occurring under the age of five[2, 3]. The incidence of HB in males is higher than in females with a reported male to female ratio ranging from 1.2 to 3.3[4].

Several syndromes are associated with a higher incidence of HB. Familial adenomatous polyposis coli (FAP) is a syndrome caused by the mutation of the adenomatous polyposis coli (*APC*) gene and is characterized by early development of colon cancer[5]. Children with FAP are at higher risk of developing HB especially in the first four years of their lives compared to the general population[6].

Beckwith–Wiedemann Syndrome (BWS) is an overgrowth syndrome caused by imprinting defects at the loci of several genes[7]. Patients usually present with large birth weight, macroglossia, and large abdominal organs. BWS is associated with a number of embryonal malignancies such as Wilms tumor, neuroblastoma and HB[8].

Various other syndromes are also associated with increased HB incidences and include trisomy 18, Simpson–Golabi–Behmel syndrome, Prader–Willi syndrome, Sotos syndrome, Kabuki syndrome, Neurofibromatosis type 1, Fanconi Anemia, Tyrosinemia type 1, Noonan syndrome and DiGeorge syndrome[9]. The molecular mechanisms by which those syndromes might promote HB development remain poorly understood.

Several studies also looked for environmental risk factors during pregnancy that might be triggering HB development. The only consistent factors found to be associated with an increased HB incidence were low birth weight and prematurity[9, 10].

### 2.1.2 Clinical presentation and diagnosis

Patients with HB commonly present with abdominal distension or a palpable abdominal mass. More general symptoms include fatigue, abdominal pain and discomfort, loss of appetite, failure to thrive and occasionally jaundice[11, 12]. Ultrasonography usually reveals a large hepatic mass and a subsequent abdominal MRI is able to expose the full extend of tumor growth. Additional imaging is performed to look for metastasis, which are found almost exclusively in the lungs[13]. In at least 70% of HB patients elevated serum alpha feto protein (AFP) level can be detected. AFP serves as a useful marker for diagnosis and risk stratification and is also used for monitoring tumor response to therapy. Depending on the risk stratification of the patients (see below) a percutaneous biopsy of the liver mass or metastatic lesions might be appropriate[4, 11].

### 2.1.3 Histology

HB is an embryonal tumor that is believed to originate from early hepatocyte precursor cells[14]. Its histological patterns resemble various stages of liver development and can be divided in two major histological subtypes[2]. The more predominant epithelial subtype makes up about 56% of HBs and can be further subdivided into pure fetal, embryonal, macrotrabecular, small cell undifferentiated and cholangioblastic[4]. The mixed subtype makes up 44% of HBs and comprises epithelial and mesenchymal features. Tumors of the mixed subgroup can contain stromal derivatives or display teratoid features. Histopathological characterization of HB tissue is not only important for diagnostic purposes, but is also prognostically relevant[15]. Although tumors are rarely composed of only one histological type it can be stated, that tumors displaying largely fetal histology are generally associated with better outcomes than those with predominant embryonal or small cell undifferentiated features[16].

### 2.1.4 Genetic and epigenetic background

Besides histology HB also has distinct molecular features that can be used for further characterization. Embryonal malignancies such as HB are believed to arise from primordial cells that are unable to reach their terminal differentiation due to genetic and epigenetic events occurring during early organ development[17]. Those molecular defects lead to developmental errors eventually amounting to the formation of an embryonal tumor.

Apart from the molecular aberrations that go along with the various above-mentioned syndromes associated with HB, there are several other defining features commonly found in HB. One of the hallmark cytogenetic findings include whole-chromosome aneuploidy with frequent additions of chromosomes 2, 8 and 20 and loss of chromosomes 4 and 18[18]. In addition an unbalanced recurring translocation has been described as der(4)t(1;4)(q12;q34) in several HB cases[19].

Furthermore, there are a number of acquired single gene alterations commonly found in HB. The most frequently mutated gene is *CTNNB1*, which encodes for the protein beta catenin, a key component of the Wnt signaling pathway[20]. This pathway plays a crucial role in early liver development. The *CTNNB1* mutations prevent beta catenin degradation, therefore leading to aberrant activation of Wnt signaling and promoting the tumorigenesis of HB[21, 22].

Another recurring feature of HB are *APC* mutations which can either occur as germ-line mutations in the context of FAP as mentioned above or appear independently as somatic mutations[23]. APC is involved in beta catenin regulation and inactivating mutations in the *APC* gene prevent beta catenin degradation, leading to a similar activation of Wnt signaling as with *CTNNB1* mutations[24].

Less common are mutations in AXIN1 and AXIN2, which are known to interact with both APC and beta catenin, therefore also influencing Wnt signaling[25].

In addition to those more or less frequently detected mutations, there are a number of further alterations on a single case level[26, 27].

Aside from genetic events, epigenetic aberrations seem to play a crucial role in HB. One commonly found alteration is the hypermethylation of gene promoters including secreted frizzled-related protein 1 (*SFRP1*), hedgehog-interacting protein (*HHIP*) and insulin-like

growth factor binding protein 3 (*IGFBP3*)[28-30]. The silencing of those tumor suppressor genes through epigenetic mechanisms leads to activation of several developmental pathways contributing to HB tumorigenesis.

### 2.1.5 Staging and molecular risk stratification

There are currently four major trial groups with different staging systems for HB. The International childhood liver tumors strategy group (SIOPEL), Children's Oncology Group (COG), the German Society for Pediatric Oncology and Hematology (GPOH), and the Japanese Study Group for Pediatric Liver Tumors (JPTL). Recently those four groups formed a global coalition called the Children's Hepatic tumors International Collaboration (CHIC) and attempted to create a consensus approach to staging and risk stratification for HB[10]. Several prognostic relevant features were identified.

Pretreatment extension of disease (PRETEXT) is a system that identifies four stages (PRETEXT I-IV), which describe the involvement of liver sections by the tumor and the tumor extension beyond the liver assessed by diagnostic imaging. PRETEXT I-III are more localized stages, generally associated with favorable outcomes, while PRETEXT IV represents advanced tumor extension as a feature of high-risk HB[31].

There is also consensus that very low AFP levels of less than 100 ng/mL and patients older than 3 years at the time of diagnosis constitute additional high-risk characteristics of HB. Metastatic disease and portal or hepatic venous macrovascular involvement are also associated with poor patient outcome[10].

Aside from those traditional staging criteria, molecular markers and gene expression signatures have shown great value in the stratification of HB. In particular, a 16-gene classifier has been shown to be equally capable in predicting HB patient outcome compared to stratification based on clinicopathological characteristics[32]. The classifier recognizes two distinct subclasses of HB. The standard-risk C1 subclass goes along with a less aggressive phenotype and favorable patient outcome. The high-risk C2 subclass is associated with advanced tumor stage, metastases, vascular invasion and poor prognosis. Although the 16-gene classifier has shown great potential in predicting outcomes, it is not yet routinely

used for risk stratification, mostly because the majority of patients are stratified and treated preoperatively without undergoing percutaneous biopsies. However, as more robust and predictive molecular markers are discovered they are likely to play a central role in future stratification and treatment strategies.

### 2.1.6 Treatment regimens and patient outcome

Over the last decades several treatment regimens for HB have been studied within clinical trials conducted by the four major trial groups mentioned above[15]. While those groups still utilize different treatment protocols some general statements about those regimens can be made. Patients are usually treated with neoadjuvant chemotherapy followed by surgical resection and adjuvant chemotherapy[11]. The chemotherapy usually has a platinum-based backbone (cisplatin or carboplatin), which can be sufficient as a monotherapy for standard-risk patients. High-risk patients are given additional chemotherapy, mainly anthracyclines such as doxorubicin. Patients presenting with unresectable tumors might qualify for liver transplantation.

Those treatment strategies have improved patient outcomes tremendously over the last decades. However, even though standard-risk patients have excellent outcomes with a three-year overall survival rate of 95%, high-risk patients presenting with advanced tumor stages, vascular invasion and distant metastases still face overall survival rates of under 60% even with aggressive treatment regimens[33, 34].

Besides the general risks of tumor resection, there are common side effects from chemotherapy. The dose-limiting factor of cisplatin is mostly ototoxicity and nephrotoxicity, sometimes with irreversible organ damage[35, 36]. Even more severe are the side effects of anthracyclines in young children. Patients treated with doxorubicin are not only at risk for acute cardiotoxicity, they might also develop doxorubicin induced cardiomyopathy years after the last dose was administered[37, 38]. This late developed cardiomyopathy is usually refractory to common medications and carries a very poor prognosis[39].

## 2.2 Goals and scope of this study

This dissertation comprises two publications both addressing fundamental questions concerning HB. The first project aimed to further examine the genetic background of HB and shed light onto molecular mechanisms driving this malignancy. The second project used a bioinformatic approach to identify new drug targets in high-risk HB and advance patient outcome through novel treatment options. The scope of both projects is outlined in brief below.

### 2.2.1 The genomic landscape of hepatoblastoma and their progenies with HCC-like features

In order to gain further insights into the origin and the genetic background of HB, we conducted whole-exome sequencing of 15 HB samples and three samples of so-called transitional liver cell tumors (TLCT) as well as matched normal liver tissues. TLCT usually occur in older children, have a particularly poor outcome and are thought to be in a transitional state between HB and hepatocellular carcinoma (HCC) with distinct differences in morphology, immunophenotype and response to treatment[40].

We found HB to have a very simple genetic background with a surprisingly low mutation rate of only 2.9 mutations per tumor. Previously described *CTNNB1* mutations were detected in 12 of 15 HB samples. We found mutations in the nuclear factor (erythroid-derived 2)-like 2 (*NFE2L2*) gene as the only other recurrent event with mutations in 2 out of 15 HB samples.

TLCT had a much more complex genetic background with an average of 27.3 mutations per tumor, which roughly compares to the mutation frequency in adult HCC[41, 42]. All three TLCTs also had *CTNNB1* mutations. Notably two TLCTs had mutations in the promoter of the telomerase reverse-transcriptase (*TERT*) gene.

When we looked for chromosomal gains and losses we found the results to be largely in agreement with previous findings. HBs showed gains at chromosomes 1, 2, 8 and 20 and losses at chromosomes 4 and 11. TLCTs showed extreme chromosomal instability, which

might be explained by the deletion or mutation of the gate keeper genes *RAD17*, *MSH6*, and *TP53*.

Other non-recurrent mutations included mostly genes involved in transcriptional regulation or chromatin organization. This partially explains why only so few mutations are needed in order to lead to an aggressive neoplasm like HB. Disrupting the transcriptional machinery or epigenetic chromatin regulation by mutations of key regulators has widespread consequences on transcriptional programs and gene expression.

To investigate whether the three recurrent mutations we found via whole-exome sequencing, namely CTNNB1, NFE2L2 and TERT, are also present in a larger cohort of patients we performed targeted sequencing of those genes in 33 additional primary tumors and cell lines. In the total cohort of now 51 cases we found *CTNNB1*, *NFE2L2* and *TERT* mutations in 72.5%, 9.8% and 5.9% of cases, respectively.

Interestingly *TERT* mutations exclusively occurred in TLCTs, making it a potential marker for the detections of high-risk patients. Mutations in the *TERT* promoter also lead to a significant overexpression of *TERT* in TLCTs compared to normal liver tissue. Notably, HBs also showed a significant, however slightly less pronounced overexpression of *TERT*.

Since we detected mutations of the transcription factor NFE2L2 in four HBs and one HB cell line, we set out to further explore the impact of those mutations on tumorigenesis. All mutations compromised the KEAP1/CUL3 binding site of NFE2L2, potentially interfering with KEAP1-mediated degradation of NFE2L2. By performing transcriptional reporter assays we found that most of the mutations lead to increased *NFE2L2* transcriptional activity, insensitive to KEAP1-mediated inhibition.

To determine whether NFE2L2 activation is also present in tumors without *NFE2L2* mutations, we measured expression levels of *NQO1* in 47 primary tumor samples. *NQO1* is a known NFE2L2 target gene, which has been shown to closely correlate with NFE2L2 activity[43]. We found *NQO1* significantly upregulated in liver tumor samples compared to normal liver tissues. More importantly, high *NQO1* expression was significantly associated with metastases, vascular invasion and the high-risk C2 subclass described above. In accordance with this, patients with high *NQO1* expression had significantly worse outcomes in terms of specific survival.

While the exact molecular mechanism by which NFE2L2 activation contributes to HB-tumorigenesis remains largely elusive, using *NQO1* as a surrogate marker to measure NFE2L2 activation in tumors might be of prognostic significance for HB patients.

### 2.2.2 Connectivity map identifies HDAC inhibition as a treatment option of high-risk hepatoblastoma

As described above, outcomes of HB patients have drastically improved with current treatment regimens. However, there is still a subgroup of high-risk patients whose outcome remains poor. In addition, the aggressive chemotherapy regimen to which high-risk patients are submitted often result in severe late effects in the surviving children, as outlined in the first part of this introduction. Therefore, new targeted treatment strategies are needed to improve patient outcome and prevent long-term side effects from conventional chemotherapy.

Since gene expression signatures have proven useful for HB risk stratification in the past we used expression data from 7 primary HBs and built a gene signature comprising the 1,000 best discriminating genes between standard-risk C1 and high-risk C2 tumors as defined by the 16-gene HB classifier described above. We used this signature as input for the Connectivity Map, a biomedical software tool, which is able to predict drugs potentially capable of inducing or reversing gene expression profiles. When we filtered the results for drugs that could potentially reverse the high-risk C2 signature, the HDAC-inhibitor SAHA (vorinostat) ranked first in our list.

Histone deacytelases (HDACs) are epigenetic chromatin modifiers that are able to inhibit transcription by promoting the formation of heterochromatin. This often leads to aberrant silencing of tumor suppressor genes and contributes to the development of various tumor entities[44]. Since high HDAC expression levels are a common feature in many cancers and have also been suggested as a positive predictor for the efficacy of HDAC inhibition (HDACi)[45, 46], we examined HDAC expression levels in 35 primary HBs and cell lines and compared them to the expression in normal liver tissue. HDAC 1, 2 and 4 were generally overexpressed in tumor tissue and cell lines. Interestingly, we found a significant correla-

tion between high expression levels of HDAC 1 and 2 and tumors exhibiting the high-risk C2 signature. Notably, overexpression of HDAC 1 and 2 have been described as a marker associated with poor prognosis in other solid tumors[47, 48].

To evaluate HDACi as a potential treatment option for HB we tested the effect of two HDAC inhibitors on liver tumor cell lines. The pan HDAC inhibitor SAHA was able to potently reduce cell viability in a dose dependent manner, while the subclass HDAC inhibitor MC1568 had only minor effects on cells. When we investigated how HDAC inhibitors conveyed growth inhibition, we found that SAHA treatment led to strong induction of apoptosis in HB cells, while cell cycle progression appeared to be unaffected. Further analysis showed a strong re-expression of hedgehog-interacting protein (*HHIP*), secreted frizzeled-related protein 1 (*SFRP1*) and insulin-like growth factor-binding protein 3 (*IGFBP3*) upon HDACi. As mentioned above, those three tumor suppressor genes are known to be epigenetically silenced in HB. These findings suggest a functional connection between re-expression of HB specific tumor suppressor genes and the apoptotic effect of HDACi.

Since the Connectivity Map predicted that HDACi might be able to reverse the high-risk C2 signature we analyzed gene expression patterns in cell lines exhibiting the adverse C2 expression profile before and after HDACi. In agreement with the Connectivity Map analysis we found a major shift in gene expression towards the standard-risk C1 signature in HB cells treated with HDAC inhibitors. As standard-risk tumors are more susceptible to conventional chemotherapy compared to high-risk tumors, we hypothesized that HDACi might sensitize HB cells to chemotherapy, especially to cisplatin.

In order to investigate this hypothesis we combined HDAC inhibitors with cisplatin and compared the effect on HB cells with the effect of the current high-risk chemotherapy regimen, which combines cisplatin and doxorubicin. We detected strong synergies between HDAC inhibitors and cisplatin at most concentrations, while synergies between cisplatin and doxorubicin were detected for only a few concentrations. Notably, we found combinations of cisplatin and SAHA to be equally and at some concentrations even more effective in reducing cell viability when compared to combinations between cisplatin and doxorubicin. These findings indicate that HDACi is in fact capable of sensitizing HB cells to cisplatin.

While further studies are needed to evaluate HDACi as a targeted treatment option in vivo, it holds the potential to replace cardiotoxic anthracyclines in high-risk treatment protocols and reduce the cumulative dose of oto- and nephrotoxic cisplatin due to its synergistic effects, without compromising treatment efficacy.

## 2.3 Contribution

The doctoral candidate Alexander Beck contributed to the publication “The genomic landscape of hepatoblastoma and their progenies with HCC-like features” by conducting experiments concerned with the identification and validation of mutations in additional primary tumor samples that were not submitted to whole-exome sequencing. Furthermore, he was involved in the functional characterization of candidate genes initially identified by whole-exome sequencing. Mr. Beck also contributed in the drafting of the manuscript.

For the publication “Connectivity map identifies HDAC inhibition as a treatment option of high-risk hepatoblastoma” the doctoral candidate conducted the bioinformatic analyses that lead to the identification of several compounds for the potential treatment of high-risk HB. He planned and performed the vast majority of experiments and carried out the statistical analysis of clinical data. He also wrote the manuscript and created all of the figures.

# 3. Summaries

## 3.1 Summary in English

Hepatoblastoma (HB) is a rare pediatric tumor, almost exclusively occurring in children under the age of five years. This embryonic malignancy is thought to arise from early hepatocyte precursor cells unable to reach their terminal differentiation due to genetic and epigenetic events disturbing normal organ development. While some of those events are well documented the exact mechanisms driving HB tumorigenesis remain largely unknown.

Over the last few decades several clinical trials were able to identify promising treatment regimens for HB patients. Common treatment protocols involve neoadjuvant chemotherapy followed by surgical resection and adjuvant chemotherapy. Standard-risk patients achieve excellent outcomes with this treatment, with a three-year overall survival rate of 95%. Unfortunately, the outcome for high-risk patients presenting with vast tumor extensions, distant metastases and vascular invasion remains poor. In addition, the cytotoxic agents utilized in HB treatment protocols can cause severe adverse effects including irreversible and potentially life threatening organ damage.

The research presented in this dissertation attempts to elucidate the genetic events in HB and the molecular mechanisms driving this malignancy. It also aims at identifying novel drug targets especially in high-risk patients with the goal of improving patient outcomes and reducing side effects from conventional chemotherapeutic agents.

Achieving those objectives involved whole-exome sequencing of primary HB samples, which revealed a very simple genetic background of only 2.9 mutations per tumor. We found recurring mutations in *CTNNB1*, *NFE2L2* and *TERT*, the latter of which were exclusively present in so-called transitional liver cell tumors (TLCT) and could represent a characterizing marker for this rare subgroup of HB. Mutations in the transcription factor NFE2L2 rendered it insensitive to proteasomal degradation, leading to increased transcriptional activity. This increased activity was also detected in HBs with no *NFE2L2* mutations as assessed by the expression of the NFE2L2 target gene *NQO1*, an established surrogate marker of NFE2LE activity. Overexpression of *NQO1* in HB was significantly associated with metastasis, vascular invasion, the adverse prognostic C2 gene signature and poor outcome, making *NQO1* a potential biomarker for risk-stratification.

In order to find new targeted therapies for high-risk HB we used a bioinformatic approach and identified HDAC inhibitors as a promising therapy option. Subsequent expression analysis showed overexpression of several HDAC subclasses in primary tumors and HB cell lines, which has been suggested to be predictive for the efficacy of HDAC inhibition. Treatment of HB cells with HDAC inhibitors resulted in potent growth inhibition, strong induction of apoptosis and re-expression epigenetically silenced tumor suppressor genes. HDAC inhibition also shifted the transcriptional program in HB cells from a high-risk expression profile to a more standard-risk expression signature. Combination of HDAC inhibitors and cisplatin showed strong synergies, which lead to efficacious reduction of HB cell viability, even at very low cisplatin doses. Our findings suggest HDAC inhibition as a novel treatment option for high-risk HB that holds the potential to reduce doses of conventional chemotherapeutic agents without compromising efficacy.

## 3.1 Summary in German

Das Hepatoblastom (HB) ist ein seltener pädiatrischer Tumor, der fast ausschließlich bei Kindern im Alter von unter fünf Jahren auftritt. Man nimmt an, dass dieser embryonale Tumor aus Hepatozyten-Vorläufern entsteht, die sich nicht richtig differenzieren können, da genetische und epigenetische Ereignisse die normale Organentwicklung stören. Obwohl einige dieser Ereignisse gut dokumentiert sind, bleiben die genauen Mechanismen, welche die Tumorgenese des HB vorantreiben weitgehend unbekannt.

Während der letzten Jahrzehnte gelang es durch zahlreiche klinische Studien vielversprechende Behandlungsmöglichkeiten für HB Patienten zu identifizieren. Herkömmliche Behandlungsprotokolle beinhalten eine neoadjuvante Chemotherapie, gefolgt von einer chirurgischen Resektion und einer adjuvanten Chemotherapie. Mit diesem Therapiekonzept erzielt man bei Standardrisiko-Patienten hervorragende Erfolge mit 3-Jahres-Überlebensraten von 95%. Leider ist das Outcome von Hochrisiko-Patienten mit ausgedehntem Tumorbefall, Metastasen und Gefäßinfiltration nach wie vor schlecht. Hinzukommt, dass die zytotoxischen Substanzen, die in der HB Therapie zum Einsatz kommen, schwere Nebenwirkungen verursachen können, einschließlich irreversibler und potential lebensbedrohlicher Organschäden.

Die vorgelegte Arbeit zielt darauf ab, genetische Veränderungen im HB eingehender zu untersuchen und molekulare Mechanismen aufzudecken, welche zur Tumorentstehung beitragen. Sie beschäftigt sich auch mit der Identifizierung von gezielten Behandlungsmöglichkeiten insbesondere für Hoch-Risikopatienten mit dem Ziel, das Outcome dieser Patientengruppe zu verbessern und Nebenwirkungen durch konventionelle Chemotherapeutika zu reduzieren.

Die Exom-Sequenzierung von HB Tumorgewebe offenbarte einen erstaunlich simplen genetischen Hintergrund mit durchschnittlich 2,9 Mutationen pro Tumor. Wir fanden wiederkehrende Mutationen in den Genen *CTNNB1*, *NFE2L2* und *TERT*, wobei letztere ausschließlich in sogenannten transitionalen Leberzelltumoren (TLCT) auftraten und einen charakteristischen Marker für diese seltene Untergruppe des HB darstellen könnten. Mutationen im Transkriptionsfaktor NFE2L2 beeinträchtigten dessen proteosomalen Abbau und erhöhten so dessen transkriptionelle Aktivität. Diese erhöhte Aktivität war auch in Tumoren nachweisbar, die keine *NFE2L2* Mutation aufwiesen und konnte über das NFE2L2

Zielgen *NQO1* nachgewiesen werden, dessen Expression ein etablierter Surrogatmarker der NFE2L2 Aktivität ist. Die Überexpression von *NQO1* in HB Gewebe war zudem signifikant mit dem Auftreten von Metastasen, Gefäßinfiltration und der ungünstigen C2 Gensignatur assoziiert. Entsprechend war eine hohe *NQO1* Expression auch mit einem schlechten Patienten-Outcome verbunden, weshalb *NQO1* einen potentiellen Biomarker zur Risikostratifizierung darstellt.

Um neue gezielte Behandlungsmöglichkeiten für Hochrisiko-HB-Patienten zu finden, verfolgten wir einen bioinformatischen Ansatz und identifizierten so HDAC Inhibitoren als eine vielversprechende Therapieoption. Eine darauffolgende Expressionsanalyse zeigte die Überexpression von mehreren HDAC-Untergruppen in primärem HB Gewebe und Tumorzelllinien. Die Überexpression von HDACs gilt als prädiktiver Marker für die Wirksamkeit von HDAC Inhibitoren. Die Behandlung von HB Zellen mit solchen HDAC Inhibitoren führte zu einer wirkungsvollen Wachstumshemmung, einer starken Induktion von Apoptose und der Reaktivierung von epigenetisch stillgelegten Tumor-Suppressor-Genen. Die HDAC Inhibition führte außerdem zu einer Verschiebung des Transkriptionsprogramms in HB Zellen von einem Hochrisiko-Expressions-Profil zu einer eher dem Standardrisiko entsprechenden Expressions-Signatur. Die Kombination von HDAC Inhibitoren und Cisplatin zeigte starke Synergien, welche selbst bei sehr niedriger Cisplatin-Dosierung zu einer wirksamen Reduktion der HB-Zellviabilität führten. Unsere Ergebnisse sprechen für den Einsatz von HDAC Inhibitoren als neuartige Therapieoption bei HB-Patienten der Hochrisikogruppe mit dem Potential die Gesamtdosis konventioneller Chemotherapeutika zu reduzieren, ohne die Wirksamkeit der Therapie zu gefährden.

# 4. Original Articles

## 4.1 Publication I

Eichenmuller M., Trippel F., Kreuder M., **Beck A.**, Schwarzmayr T., Häberle B., Cairo S., Leuschner I., von Schweinitz D., Strom T. M., Kappler R. *The genomic landscape of hepatoblastoma and their progenies with HCC-like features.* Journal of hepatology 2014; 61:1312-20 (Impact (2014)=11.336)

Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

# The genomic landscape of hepatoblastoma and their progenies with HCC-like features

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See Editorial, pages 1202–1204

**Background & Aims**: Hepatoblastoma (HB) is the most common childhood liver cancer and occasionally presents with histological and clinical features reminiscent of hepatocellular carcinoma (HCC). Identification of molecular mechanisms that drive the neoplastic continuation towards more aggressive HCC phenotypes may help to guide the new stage of targeted therapies.
**Methods**: We performed comprehensive studies on genetic and chromosomal alterations as well as candidate gene function and their clinical relevance.
**Results**: Whole-exome sequencing identified HB as a genetically very simple tumour (2.9 mutations per tumour) with recurrent mutations in ß-catenin (*CTNNB1*) (12/15 cases) and the transcription factor *NFE2L2* (2/15 cases). Their HCC-like progenies share the common *CTNNB1* mutation, but additionally exhibit a significantly increased mutation number and chromosomal instability due to deletions of the genome guardians *RAD17* and *TP53*, accompanied by telomerase reverse-transcriptase (*TERT*) promoter mutations. Targeted genotyping of 33 primary tumours and cell lines revealed *CTNNB1*, *NFE2L2*, and *TERT* mutations in 72.5%, 9.8%, and 5.9% of cases, respectively. All *NFE2L2* mutations affected residues of the NFE2L2 protein that are recognized by the KEAP1/CUL3 complex for proteasomal degradation. Consequently, cells transfected with mutant *NFE2L2* were insensitive to KEAP1-mediated downregulation of NFE2L2 signalling. Clinically, overexpression of the NFE2L2 target gene *NQO1* in tumours was significantly associated with metastasis, vascular invasion, the adverse prognostic C2 gene signature, as well as poor outcome.
**Conclusions**: Our study demonstrates the importance of *CTNNB1* mutations and NFE2L2-KEAP1 pathway activation in HB development and defines loss of genomic stability and *TERT* promoter mutations as prominent characteristics of aggressive HB with HCC features.




## Introduction

Hepatoblastoma (HB) is a highly malignant liver tumour, arising in children under the age of three years, with a histology that resembles various stages of the developing liver comprising epithelial phenotypes (less differentiated embryonal and differentiated foetal) and mesenchymal elements such as immature fibrous tissue or osteoid [1]. Occasionally, an aggressive subtype of HB presents in children >5 years of age with clinical and histopathological features reminiscent of hepatocellular carcinoma (HCC) [2]. This so-called transitional liver cell tumour (TLCT) has been suggested to indicate a neoplastic continuation along an ontogenetic differentiation pathway from HB to HCC [2].

Based on its early manifestation it is generally assumed that HB displays a relatively normal genomic background, which is reflected by the detection of only a few cytogenetic and genetic alterations [3]. So far, mutation of ß-catenin (*CTNNB1*), the key molecule of Wnt signalling, represents the only recurrent alteration found in about two thirds of HB patients, mainly hitting exon 3 either by point mutation or deletion [4]. This leads to the disruption of phosphorylation sites, which are needed to

Keywords: Hepatoblastoma; Exome; Beta catenin; NFE2L2; Mutation; Telomerase reverse-transcriptase; Hepatocellular carcinoma; Paediatric.
*Received 28 March 2014; received in revised form 15 July 2014; accepted 7 August 2014; available online 15 August 2014*
☆ DOI of original article: http://dx.doi.org/10.1016/j.jhep.2014.09.016.
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*Abbreviations:* HB, hepatoblastoma; HCC, hepatocellular carcinoma; CTNNB1, beta catenin; NFE2L2, nuclear factor (erythroid-derived 2)-like 2; TERT, telomerase reverse-transcriptase; NQO1, NAD(P)H: quinone oxidoreductase 1; KEAP1, kelch-like ECH-associated protein 1; CUL3, cullin 3; TLCT, transitional liver cell tumour; APC, adenomatous polyposis coli; PRETEXT, pretreatment extent of disease; PCR, polymerase chain reaction; indel, insertion and deletion; CNV, copy number variations; ARE, antioxidant response element; TBP, TATA-box binding-protein; SD, standard deviation; SEM, standard error of the mean.

tag CTNNB1 for proteasomal degradation by a destruction complex consisting of the adenomatous polyposis coli (APC) protein, AXIN, glycogen synthase kinase 3, and casein kinase 1. Consistently, somatic mutations of *APC* as well as *AXIN1* and *AXIN2* have been found in HB, although at a very low frequency [5,6]. Of note, genetic lesions in *CTNNB1*, *AXIN1*, and *AXIN2* are similarly observed in adult HCC [6,7]. These data clearly underscore the significance of activated Wnt signalling in the genesis of liver cancer, both in very young children and adults [8].

Based on recent transcriptional data, HB can be distinguished into two distinct groups: the so-called C1 subclass that recapitulates liver features at late stages of intrauterine life with a mostly foetal histotype and the expression of markers of mature hepatocytes, and the C2 subclass that resembles earlier stages of liver development with a predominantly embryonal histotype and the expression of markers of hepatic progenitor cells, such as cytokeratin 19 and EpCAM [9]. Most importantly, a specific 16-gene signature has been deduced from these profiling experiments that predicts outcome of HB patients better than classical criteria, such as tumour stage by pretreatment extent of disease (PRETEXT), vascular invasion, and extrahepatic metastases. Besides this characteristic expression profile, HB displays a general overexpression of several developmental genes, including the imprinted genes *IGF2* [10] and *DLK1* [9] as well as genes involved in the hedgehog pathway [11]. Although the cause for the upregulation of these genes is still enigmatic, epigenetic mechanisms such as loss of imprinting and promoter hypermethylation have been emphasized [3].

The aim of this study was to further our understanding of the genetic basis of HB and to identify molecular mechanisms that drive a neoplastic continuation towards HCC by applying whole-exome sequencing.

## Materials and methods

### *Patients and materials*

A total of 47 liver tumour specimens were obtained from paediatric patients undergoing surgical resection in our department. Written informed consent was obtained from each patient and the study protocol was approved by the local authorities. Furthermore, we used the four human HB cell lines HepT1, HepT3 (both provided by Dr. T. Pietsch), HuH6 (Japanese Collection of Research Bioresources, Osaka, Japan), and HepG2 (ATCC, Manassas, VA, USA), as well as the HEK293T cell line (ATCC). Tumour samples and HB cell lines were assigned to C1 and C2 subtypes using the 16-gene classifier as reported previously [9]. Human *NFE2L2* cDNAs (wild type and mutant forms) were subcloned into the pEGFP-N1 vector (Clontech, Mountain View, CA) by polymerase chain reaction (PCR); cloning and sequence was verified by Sanger sequencing. The pFLAG-KEAP1 expression construct and the pNQO1-ARE luciferase reporter vectors have been described previously [12,13].

### *Exome sequencing*

All tumour samples were clinically and pathologically well-characterized and macro-dissected to enrich for neoplastic cellularity. Genomic DNA of 18 fresh-frozen tumour specimens and matched healthy liver tissues was extracted with the DNeasy blood and tissue Kit (Qiagen, Hilden, Germany). Quality was checked by electrophoresis in a 1% agarose gel and quantitatively measured using the NanoDrop 1000 instrument (Thermo Scientific, Wilmington, DE, USA).

Exomes were enriched in solution with SureSelect XT Human All Exon 50 Mb kits (Agilent Technologies, Santa Clara, CA, USA) and sequenced as 100 bp paired-end runs on a HiSeq2500 system (Illumina, San Diego, CA, USA) generating 7–14 Gb of sequence and an average read depth between 87 and 172 on target regions. More than 90% of the target regions were covered 20 times or more. Burrows-Wheeler Aligner (BWA v 0.5.9) with standard parameters was used for read alignment against the human genome assembly hg19 (GRCh37). We performed single-nucleotide variant and small insertion and deletion (indel) calling for the regions targeted by the exome enrichment kit using SAMtools (v 0.1.18). Large indels were called with Pindel (v 0.2.4t) and copy number variations (CNV) were determined using the R package ExomeDepth (v 0.9.7). Allelic imbalances were determined by calculating the fraction of reads carrying the mutation in the tumour sequences at genomic positions where heterozygous SNVs were present in the control tissue.

To discover putative somatic variants, we retrieved only those variants of a tumour that were not found in the corresponding control tissue. To reduce the number of false positives, we filtered out variants that were already present in 3500 "in house" control exomes (patients with unrelated diseases and healthy controls from other projects) or had variant quality of less than 30. Furthermore, the variants were filtered according to several quality criteria using the SAMtools varFilter script. We used default parameters, with the exception of the maximum read depth (-D) and the minimum *p* value for base quality bias (-2), which we set to 9999 and 1e-400, respectively. Moreover, we applied a custom script that marked all variants where the median base quality of adjacent bases was low, because these variants are often sequencing artefacts. We then manually investigated the raw read data of the remaining variants using the Integrative Genomics Viewer (IGV v2.3.19).

### *Sanger sequencing*

Sequence verification was carried out by PCR amplification of candidate exons using High Fidelity Taq polymerase (Thermo Scientific, Schwerte, Germany) and subsequent Sanger sequencing of ExoSap-IT (Affymetrix, Santa Clara, CA, USA) purified amplicons. PCR conditions for detecting mutations in exon 2 of the *NFE2L2* gene (primers NRF2-EX2-F and NRF2-EX2-R), point mutations (primers BCAT-1 and BCAT-2) or deletions (primers BCAT-3 and BCAT-4) in exon 3 of the *CTNNB1* gene as well as *TERT* promoter mutations have been described previously [4,14,15]. Sequencing was done on an ABI 3730 capillary sequencer in the LMU sequencing facility using the ABI BigDye Terminator kit (Applied Biosystems, Foster City, CA). Sequence analysis was performed using the Staden Package 2.0 program.

### *Real-time PCR*

RNA extraction and purification, cDNA synthesis, PCR amplifications and quantization of gene expression were performed as described before [11] using the following primer pairs: *TERT*, CACGGAGACCACGTTTCAAA, GCACCCTCTTCA AGTGCTGTC; *NQO1*, GCTGCCATGTATGACAAAGGAC, CCGGTGGATCCCTTGCAGA; *TBP*, GCCCGAAACGCCGAATAT, CCGTGGTTCGTGGCTCTCT.

### *NQO1-antioxidant response element (ARE) reporter assay*

Cells were seeded in 12-well plates the day before transfection. Cells were then transfected with 200 ng of the reporter plasmid pNQO1-ARE-Luc, 200 ng expression constructs (pEGFP-N1, pEGFP-WT, pEGFP-L30P, pEGFP-R34P, pEGFP-R34G, or pEGFP-T80A), 200 ng pFLAG-KEAP1, and 10 ng of the reference plasmid pRL-CMV (Promega, Madison, Wisconsin, USA) using the XtremeGENE HP transfection reagent (Roche Diagnostics) as recommended. 48 h after transfection, cells were lysed and reporter gene activity was determined using the Dual-Glo Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity. All reporter assay experiments were repeated four times and transfections done in duplicate.

### *NFE2L2 localization analyses*

Cells were seeded onto 18 mm coverslips (Thermo Scientific, Braunschweig, Germany) the day before transfection and then transfected with the respective GFP-tagged *NFE2L2* expression constructs using XtremeGENE HP (Roche Diagnostics). After 48 h cells were washed with PBS and fixed with 3% paraformaldehyde. Processed coverslips were counterstained with Vectashield® containing 4,6-diamidino-2-pheylindole (Vector Laboratories Inc., Burlingame, CA, USA) and mounted onto glass slides. Images were acquired using the Zeiss Axiovert 135 Microscope (Zeiss, Jena, Germany).

### *Statistical analyses*

Data were expressed as means ± standard deviation (SD) or standard error of the mean (SEM) and statistically subjected to Student's unpaired *t* test and Spearman's rank correlation test. Kaplan-Meier estimates of specific survival time in the various groups were compared using the log-rank Mantel-Cox test. A level of $p < 0.05$ was considered to be significant, $p < 0.01$ highly significant.

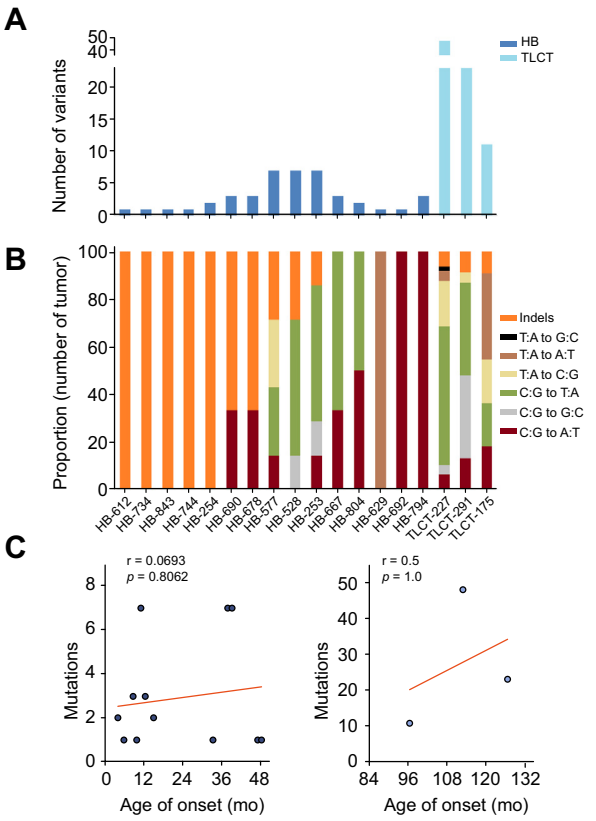


**Fig. 1. Exome sequencing of paediatric liver tumours.** (A) Distribution of somatic mutations as well as (B) frequency of insertions/deletions (indels) and nucleotide substitutions identified in 15 hepatoblastoma (HB) and 3 transitional liver cell tumour (TLCT) cases. (C) The age of disease onset of HB (left) and TLCT (right) cases was plotted against the number of somatic mutations and Spearman's rank correlation was performed, with the linear regression depicted in orange. (This figure appears in colour on the web.)

Functional annotation of mutated genes was performed using the DAVID Bioinformatics Resources v6.7 (National Cancer Institute, Frederick, MD) by computing gene-ontology statistics against the whole human genome database.

## Results

### *Hepatoblastoma harbours only few somatic mutations*

To better understand the genetic basis of childhood liver cancer, we performed whole-exome sequencing on a discovery cohort of 15 HB and three TLCT as well as matched normal liver tissues. Using stringent criteria, we identified a total of 125 somatic mutations (Supplementary Table 1). In HB, the overall mutation rate was very low with only 2.9 (range 1 to 7) variants per tumour genome (Fig. 1A), with a preponderance of deletions (Fig. 1B). Interestingly, six tumours showed only one somatic mutation, suggesting that acquisition of very few somatic mutations might be sufficient to drive tumour development in the liver. In their HCC-like progenies, the mean sequence variation rate increased to 27.3 (range 11 to 48) per tumour genome, indicating an almost comparable frequency to adult HCC that ranges from 35 to 66 [16–18]. The distribution of somatic substitutions revealed the predominance of C:G to T:A transitions in HB and TLCT (Fig. 1B), which is consistent with data on adult HCC tumours that develop on non-cirrhotic livers [17] and might be explained by spontaneous deamination [19] and/or the high GC content of coding exons [20]. Since we found no age-dependency in HB and TLCT by plotting the mutation number against the onset of the disease (Fig. 1C), other factors than the longer exposure time to genotoxic influences could have contributed to the more advanced genetic level of TLCT.

### *Increased chromosomal instability in hepatoblastoma with HCC features*

Recent studies have shown that exome sequencing can be successfully used to detect allelic imbalances and copy number variations [21], thereby enabling us to resolve chromosomal gains, losses, and copy-neutral allelic imbalances (Fig. 2A and B, Supplementary Table 2). Frequent gains were found at chromosome 2 (11/18), 1q (8/18), 20 (6/18), 6p (4/18), 8q (4/18), 12 (4/18), and 17 (4/18), whereas recurrent losses and/or copy-neutral allelic imbalances occurred at chromosome 11p (6/18) and 4q (5/18). These results are comparable to earlier work, which also detected far more gains than losses, especially on chromosomes 2 (44%), 1q (41%), 20 (24%), and 8q, the latter two being predictive for poor outcome [22]. Of note, copy-neutral allelic imbalances on the distal part of chromosome 11p presumably reflecting loss of heterozygosity at the *IGF2/H19* locus were specific for HB, whereas TLCT showed deletions of this region.

Interestingly, the mutation rate correlated with the number of chromosomal alterations ($r = 0.7155$; $p = 0.0008$). This was especially true for the two cases TLCT-227 and TLCT-291, in which we found the highest mutation rate of the discovery set, accompanied by an extreme chromosomal instability (Fig. 2B). Accordingly, TLCT-227 harbours a small deletion on chromosome 5 encompassing the locus of the *RAD17* gene (Fig. 2C), which is known to be essential for the maintenance of chromosomal stability [23]. In addition, we found a c.3991C>T mutation in the *MSH6* gene giving rise to a premature stop codon (Supplementary Table 1). Although MSH6 is considered to exclusively play a role in microsatellite instability, a first implication in chromosomal instability has recently been demonstrated in sporadic colorectal cancer [24]. In TLCT-291 we detected a small deletion on chromosome 17 at the locus of *TP53* (Fig. 2C), a tumour suppressor involved in chromothripsis [25]. These findings suggest that mutations in genes involved in chromosomal stability might be an important mechanism to drive malignant liver cells towards a more aggressive HCC-like phenotype.

### *Transcriptional regulators are frequently mutated in childhood liver cancer*

In order to identify molecular mechanisms that are frequently hit by mutations in childhood liver cancer, we performed functional annotation of the mutated genes, using the DAVID database (Supplementary Table 3). The main biological processes detected in the 15 HB were transcription (30% of mutated genes), chromatin organization (20%), and chromosome organization (20%). Accordingly, the top-scoring cellular component was the nucleoplasm (13.3%). The most prominent candidate genes code for histone H3.1 (*HIST1H3C*), histone deacetylase 4 (*HDAC4*), lysine-specific demethylase 5C (*KDM5C*), lysine-specific methyltransferase 2C

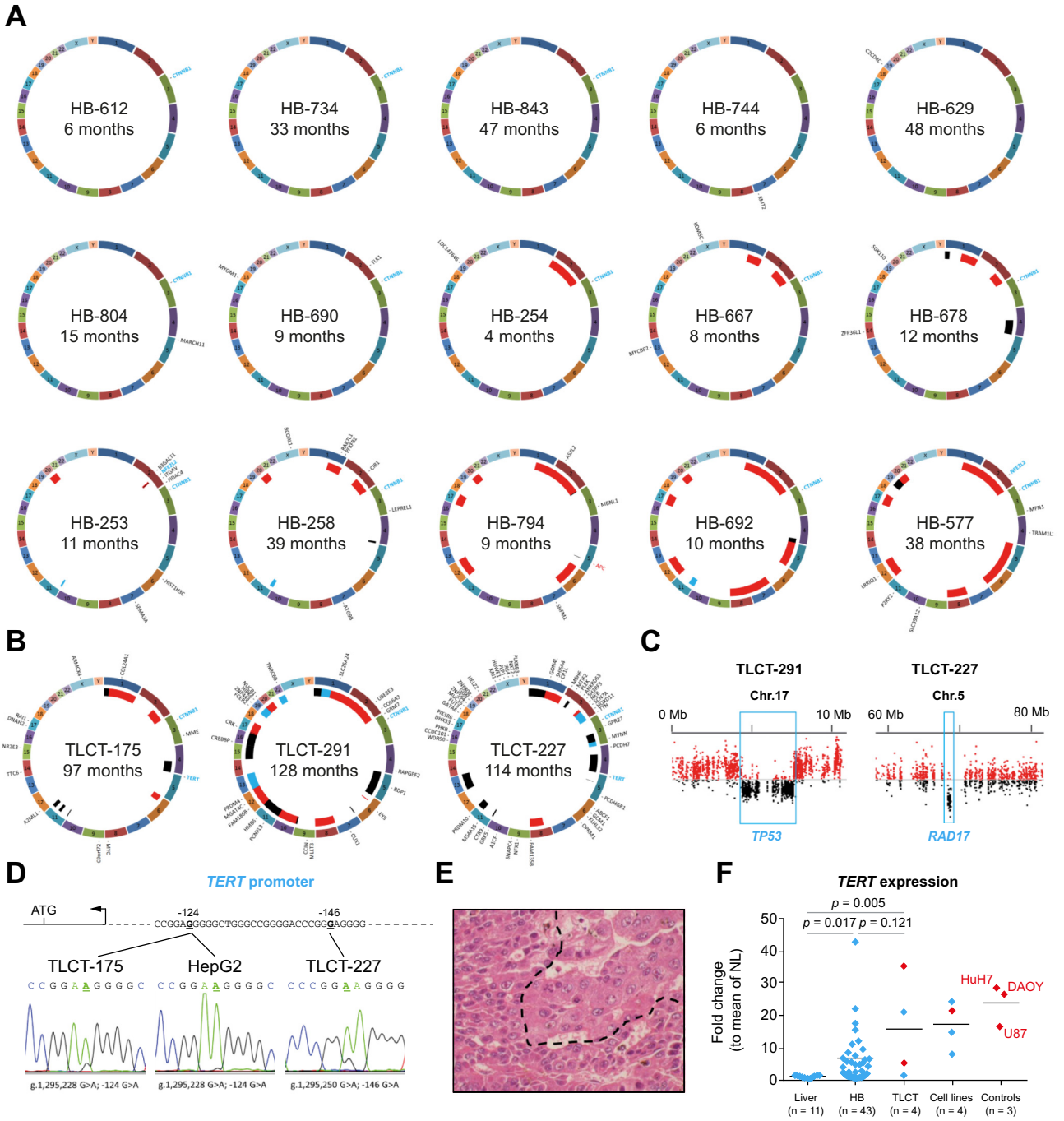


**Fig. 2. Mutations and copy number variations in paediatric liver tumours.** Ring plots of (A) HB and (B) TLCT cases with chromosomes arranged end to end in the outermost ring, and mutated genes depicted outside of each diagram (recurrent mutations are highlighted in blue font, the known germ-line *APC* mutation in HB-794 in red font). The inside ring shows somatic copy number gains (red), losses (black), and copy-neutral allelic imbalances (blue). (C) Magnification of genomic loci of two TLCT cases depicting a deletion on chromosome 17 encompassing the locus of the *TP53* gene and a small deletion on chromosome 5 at the locus of *RAD17*. Copy number variations were determined with the software ExomeDepth by comparing the number of reads per target region of the tumour and the corresponding control sample. Calculated ratios were plotted along the corresponding chromosomes as red ($\geqslant 1$) and black dots (<1) using the Integrative Genomics Viewer. (D) Schematic drawing of the *TERT* promoter region and the mutations verified by Sanger sequencing at position −124 and −146 bp upstream of the transcription start site (arrow). (E) Histological stain of TLCT-227 showing both characteristics of HB with small cells and compact nuclei (left compartment) and with large cells and pale nuclei resembling HCC (marked by dashed line on the right). (F) Relative *TERT* mRNA levels in normal liver, HB and TLCT tissues, HB cell lines as well as known *TERT* mutated tumour cell lines as controls, normalized to the mean of normal liver tissues. *TERT* mutated cases are highlighted in red. Means are given as black lines and were compared using the unpaired Student's *t* test.

(*KMT2C*), two co-repressors interacting with histone deacetylases (*CIR1*, *BCORL1*), one co-repressor binding polycomb repressive complex 2 (*ASXL2*), one E3 ubiquitin protein ligase (*MYCBP2*), as well as one transcription factor (*NFE2L2*). By looking into the TLCT candidate genes, we again found transcription and its regulation being the main biological processes. Important mutated genes comprise the co-repressor *GON4L*, two co-activators (*PRIC285*, *CCDC101*), another E3 ubiquitin protein ligase (*HUWE1*), as well as several transcription factors (*MYC*, *GATA6*, *HOXD11*, *PRDM10*, *NFX1*). These data clearly indicate that deregulation of the transcription machinery is an important step in liver cancer development.

*Activation of Wnt signalling is the key event in liver tumourigenesis*

Alteration of *CTNNB1* was the most prominent feature in paediatric liver tumours affected by either non-silent mutations (4 cases) or deletions (11 cases) of exon 3 (Supplementary Table 1). Intriguingly, three HB showed mutations in *CTNNB1* as the sole event, without any further genetic or chromosomal alteration. Additionally, we found chromosomal loss of the *APC* locus (Fig. 2A) as the second hit to the already known inherited c.3809_3810insC frame-shift mutation in the *APC* gene (Supplementary Table 1) in a 9-month-old female with familial adenomatous polyposis syndrome. These data clearly indicate that activation of the Wnt pathway is the key driver of tumourigenesis in the liver. However, two HB that lack mutations in any of the Wnt-associated genes (Fig. 2A) suggested that other molecular mechanism must exist. Indeed, we found a 506 bp deletion in the putative tumour suppressor gene *KMT2C* as the only alteration in HB-744 (Supplementary Table 1). Interestingly, *KMT2C* (alias *MLL3*) was recently identified to be mutated in a variety of cancers, including 5.4% of HCC and 14.8% of cholangiocarcinoma cases [26,27].

*Promoter mutation of TERT exclusively occurs in transitional liver cell tumours*

Previous studies have shown that HCC and their preneoplastic lesions carry specific somatic mutations in the promoter region of the telomerase reverse-transcriptase (*TERT*) gene, which could lead to transcriptional upregulation of *TERT* [15]. By visual inspection of the respective region that is not systematically covered by exome data analysis tools, we found *TERT* promoter mutations in two cases diagnosed as TLCT (Fig. 2B). Screening of a validation cohort of 33 primary tumours and cell lines by conventional Sanger sequencing revealed only one additional *TERT* promoter mutation, namely in the HepG2 cell line (Figs. 2D and 3A). Although HepG2 cells have recently been reclassified based on the original resection specimen as epithelial HB [28], it should be at least envisioned that it might be a TLCT due to the advanced age of the patient (15-year-old boy) and the fact that HB exclusively occurs in children under the age of five years [3]. Strikingly, our results resemble recent findings in adult liver tumours, which showed that hepatocellular adenoma with HCC foci, as also evident in TLCT (Fig. 2E), as well as HCC, were mutated in 44% and 59% of all cases, respectively [15]. By looking into mRNA expression we found significantly increased *TERT* levels in HB and TLCT compared to normal liver tissues (Fig. 2F). Although there was a trend towards *TERT* upregulation in *TERT* mutated cases and cell lines, no significant difference between HB and TLCT was found. Taken together, these data suggest that the *TERT* promoter mutation is a selective phenomenon of advanced HB with HCC-like features, thereby providing an excellent marker for the detection of high-risk patients.

*Recurrent NFE2L2 mutations in hepatoblastoma*

Besides *CTNNB1* and *TERT* we identified missense mutations in the nuclear factor (erythroid-derived 2)-like 2 (*NFE2L2*) gene as another recurrent event, which was affected in two HB cases (Supplementary Table 1). Targeted sequencing of our validation cohort identified missense mutations in two other HB cases and in the HepT1 cell line (Supplementary Fig. 1A), altogether in 9.8% of cases (Fig. 3A). Interestingly, the five *NFE2L2* mutations were found in cases harbouring *CTNNB1* mutations (72.5% of all cases), a coincidence already described for adult HCC [17]. *NFE2L2* mutations were located either in or adjacent to the DLG and ETGE motifs (Supplementary Fig. 1B), which have been described to be essential for binding of the KEAP1/CUL3 complex that mediates ubiquitination and proteasomal degradation of NFE2L2 [14]. In order to determine whether the identified *NFE2L2* mutations lead to NRF2 transcriptional activity that is insensitive to KEAP1-mediated degradation, we cloned the wild type and the four mutated forms of NFE2L2 (L30P, R34P, R34G, and T80A) into a GFP-tagged vector and ectopically expressed them in the presence or absence of KEAP1 in HEK293T cells that are known to exhibit low basal levels of NFE2L2-dependent transactivation [13]. Using a firefly luciferase reporter with an ARE binding sites as readout for NFE2L2 transcriptional activity [13], we found that both wild type and mutant NFE2L2 strongly increased reporter activity, which was more pronounced for the mutant forms (Fig. 3B). Co-transfection of KEAP1 resulted in a significant decrease in reporter activity in wild type *NFE2L2* and R34P transfected cells, whereas in cells transfected with the mutated forms L30P, R34G, and T80A this reduction was completely prevented. In line with this, the latter three mutated forms accumulated exclusively in the nucleus indicative for transcriptional activity, while the R34P mutant as well as wild type NFE2L2 were found both in the cytoplasm and the nucleus (Fig. 3C). Accordingly, the same sets of experiments were performed in the HB cell line HuH6, which has a comparably low NFE2L2-dependent transactivation as HEK293T, showing identical results (Supplementary Fig. 1C and D). HepG2 cells that have already a high endogenous NFE2L2 transcriptional activity followed the same trend, but without reaching statistical significance. These data clearly demonstrate that *NFE2L2* mutations found in HB result in a high NFE2L2 transcriptional activity by interfering with KEAP1-mediated degradation.

*Upregulation of the NFE2L2 target gene NQO1 is associated with poor outcome*

In order to see whether transcriptional deregulation of NFE2L2 signalling is a common phenomenon in paediatric liver cancers, we determined the expression level of the target gene *NQO1* in our cohort of 43 HB and 4 TLCT primary tumours. *NQO1* was chosen because it has been described as a reliable NFE2L2 target in *NFE2L2*-activated cancers [13,14] and outcompeted several other target genes known from non-malignant conditions by most closely reflecting the *NFE2L2* mutational status of our HB cases (Supplementary Fig. 2A) and being transcriptionally activated up to

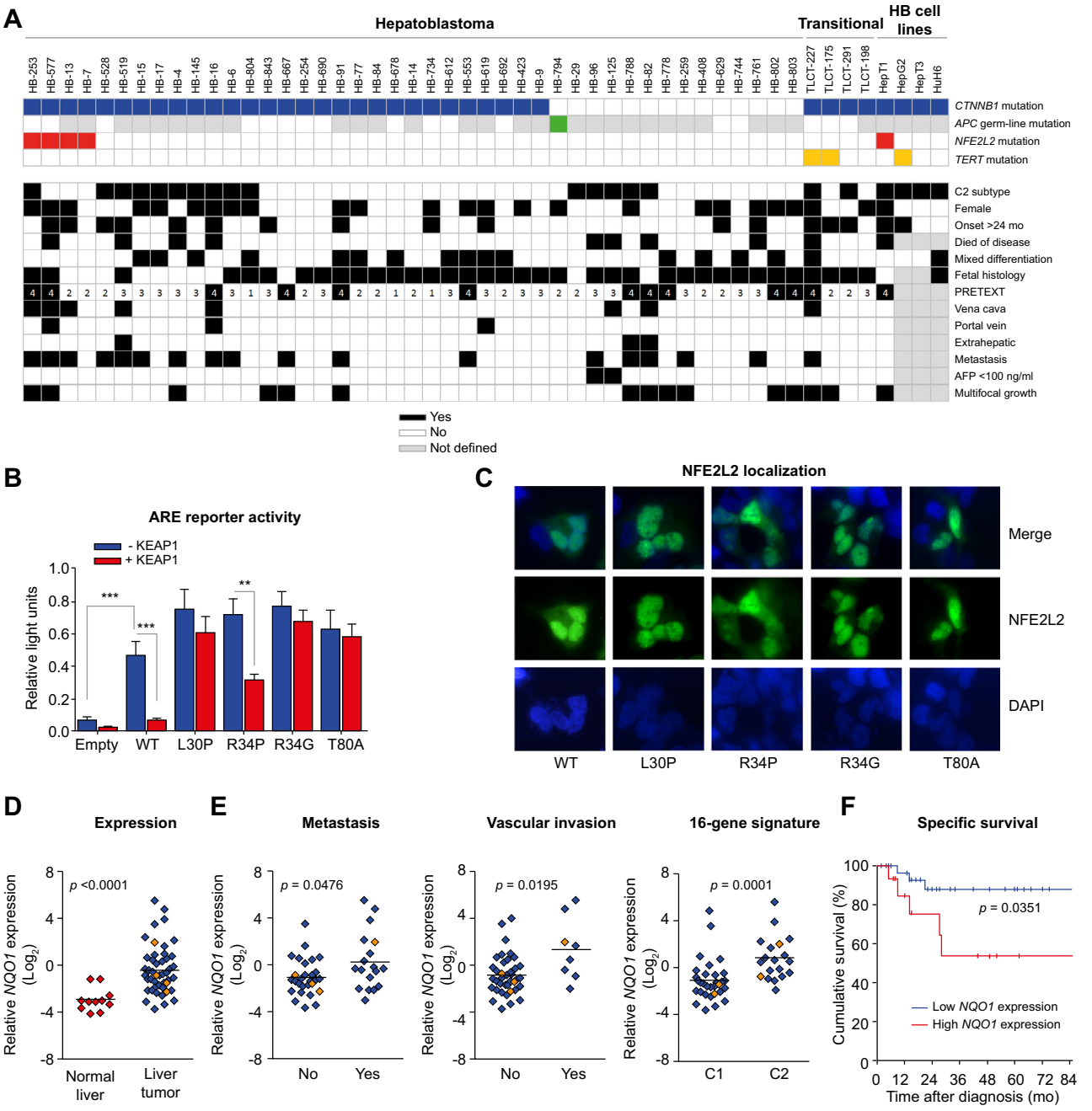


**Fig. 3. Mutational status and functional relevance of *NFE2L2* in hepatoblastoma.** (A) Clinicopathological characteristics and the mutational status of the *CTNNB1*, *APC*, and *NFE2L2* genes as well as the *TERT* promoter region are color-coded and depicted in rows for each tumour of our cohort of 43 hepatoblastoma (HB) and 4 transitional liver cell tumour (TLCT) patients and 4 HB cell lines. (B) Reporter assay experiments of HEK293T cells, transiently transfected with the pEGFP vector containing wild type (WT) or the four mutated forms of *NFE2L2*, in the presence or absence of *KEAP1*. The activity of the NFE2L2-responsive ARE luciferase reporter was measured after 48 h and normalized to the activity of Renilla luciferase. Mean ± SEM of four reporter assay experiments in duplicates are shown. (C) Exclusive nuclear accumulation of the GFP-tagged mutant NFE2L2 proteins L30P, R34G, and T80A (green) after transfection into HEK293T cells, counterstained with DAPI (blue). The R34P mutant as well as the wild type NFE2L2 protein were located both in the cytoplasm and the nucleus. (D) Expression of the NFE2L2 target gene *NQO1* relative to the housekeeping gene *TBP* in 11 normal liver (red diamonds), 43 HB (blue diamonds) and 4 TLCT tissues (orange diamonds), with the mean given as a black line. (E) Correlation of the relative *NQO1* expression of the tumour samples (TLCT highlighted in orange) with the clinical parameters metastasis, vascular invasion, and the 16-gene signature [9]. The mean expression values (black lines) and statistical significances from the unpaired Student's *t* test are given. (F) Specific survival was calculated as time from diagnosis to death of the disease and is plotted for 32 HB/TLCT patients with low (blue line) and 15 with high (red line) *NQO1* expression (defined as >5-fold increased expression than the mean of 11 normal liver tissues) over a period of 84 months. Statistical significance was calculated using the Mantel-Cox test. (This figure appears in colour on the web.)

25-fold in HB (Supplementary Fig. 2B) when looking into previously published microarray data [9]. We found a striking upregulation of *NQO1* in the tumour tissues compared to normal livers (Fig. 3D), with no main differences between HB and TLCT. Interestingly, three tumours harbouring *NFE2L2* mutation exhibited the highest *NQO1* expression. Mutations of the genes *KEAP1* and *CUL3*, which have been described to be alternatively mutated in NFE2L2-activated cancers [13,29], were ruled out by targeted sequencing of high *NQO1* expressing tumours that lack *NFE2L2* mutations, thereby suggesting alternative genetic and/or epigenetic mechanisms that drive activation of NFE2L2 signalling in paediatric liver cancers.

By correlating *NQO1* expression with clinicopathological features such as gender, onset of disease, histology, multifocal growth, outcome, and the high-risk characteristics vascular invasion (main portal vein, vena cava or three hepatic veins), intra-abdominal extrahepatic extension, metastatic disease, tumour in all liver sections (PRETEXT-IV), or alpha-fetoprotein at diagnosis less than 100 ng/ml [30], we found that *NQO1* was significantly increased in metastatic and vessel invasive tumours (Fig. 3E). Interestingly, the aggressive C2 molecular subtype, assigned by the prognostic 16-gene signature [9], was also significantly associated with high *NQO1* expression. In line with this, specific survival of patients with high *NQO1* expression was significantly worse compared to low expressers (Fig. 3F). These data suggest that NFE2L2 activation might be of prognostic significance for HB patients.

## Discussion

Our exome sequencing study provides the first comprehensive catalogue of genetic alterations in paediatric liver tumours and adds to the growing list of genomic landscapes clearly indicating that childhood cancers do not require as many genetic alterations as typical adult cancers. HB displays a genetically very simple tumour with one of the lowest mutation rates ever reported for any malignancy [31]. Contrarily, TLCT exhibited a mutation rate merely comparable to the frequency found in adult HCC [16–18], thereby implying a more advanced genetic level that could be explained by the higher degree of so-called passenger mutations in this tumour type. It is well established that passenger mutations provide an evolutionary clock that precisely records the number of DNA replications a cell has made [19]. However, we found no positive correlation between increased patients' age and mutation number, a phenomenon that has been described for medulloblastoma [32] and neuroblastoma [33]. Instead, mutations in the gate keeper genes *RAD17*, *MSH6*, and *TP53* that ensure chromosomal stability might have contributed to the higher mutation rates in TLCT, which is also known from HCC, prostate adenocarcinoma, and medulloblastoma [25,26,34]. Thus, it might be assumed that TLCT is a progeny of HB that has lost its genomic integrity, rather than an early onset HCC.

There are several details that support this hypothesis: (i) In accordance with our and previous data that mutation of *CTNNB1* is obligatory in HB pathogenesis, but not in HCC (only about 30% are mutated) [8], all four TLCT patients harboured a *CTNNB1* mutation. (ii) Except for some very rare cases (presumably misclassified rhabdoid tumours) HB patients have highly elevated AFP serum levels, as opposed to 40–60% of paediatric HCC patients [35]. The average AFP level at diagnosis of our TLCT patients was very high (217,000 ng/ml), a level comparable to those found in TLCT described earlier [2]. (iii) Histological examination of our four TLCT cases showed a characteristic HB histology with predominantly foetal differentiation in all cases, but also aspects of HCC morphology, including focal nests reminiscent of HCC (Fig. 2E). In case of TLCT-227, we analysed the local recurrence, but the initial tumour was diagnosed as a pure HB, thereby indicating that a progression towards HCC exists. (iv) TLCT share chromosomal imbalances with HB, such as the early gain of chromosomes 1 and 2 and gain of chromosome 8 in more advanced HB (Fig. 2A and B). (v) Three of the patients are still alive, which is in contrast to the poor survival rate of paediatric HCC. (vi) HB and TLCT display comparable higher *TERT* expression levels than normal liver tissue, thereby suggesting a common origin from a liver precursor cell with high self-renewal rates and thus physiologically high telomerase activity. In contrast, HCCs are thought to originate from normal hepatocytes that do not cycle often and might acquire telomerase reactivation through *TERT* mutations [15]. Being aware that we have only analysed a very limited number of TLCTs, we believe that our data in sum could support the conclusion that TLCT is a genetically derailed progeny of HB.

Our data moreover suggest that the mutation of *CTNNB1* (or alternatively *APC* or *AXIN1*) could be the sole genetic basis of liver tumours in early childhood, especially in light of three HB cases, showing mutation of *CTNNB1* as the only alteration. Comparably, retinoblastoma and rhabdoid tumours have already been shown to display a very simple genome with *RB1* [36] and *SMARCB1* [37] being the only recurrently mutated gene, respectively. However, introducing activating mutations of *Ctnnb1* into the mouse liver is not sufficient to drive tumourigenesis, giving rise only to marked hepatomegaly [38,39]. Thus, additional mutations and/or epigenetic changes may be required for HB development.

One alternative mechanism might be mutation of *NFE2L2* and more importantly activation of the NFE2L2-KEAP1 pathway. Intriguingly, comparable alterations have been detected in HCC in adults, showing either *NFE2L2* or *KEAP1* mutations in altogether 7.2% [17], 8.0% [18], and 8.9% [40] of all patients, thereby suggesting a broader role of the NFE2L2-KEAP1 pathway in liver cancers. Although the mutations found in HB render NFE2L2 insensitive to KEAP1-mediated degradation, leading to constitutive activation of the pathway, we found half of the tumour cases show pathway activation without *NFE2L2* mutations, thereby suggesting that other yet unidentified activating mechanisms must exist. Transcriptional downregulation of *KEAP1* might be one possibility, however we and others have failed to detect significant differences between normal liver and liver tumour samples, although a trend for decreased *KEAP1* expression in tumours with *KEAP1* mutations has been reported [18]. Accordingly, promoter methylation of *KEAP1*, as shown for lung cancer [41], can be dismissed too. However, as the *NFE2L2* mutated cases were concomitantly mutated in *CTNNB1*, as described before for adult HCC [17], we hypothesize that activated Wnt signalling and activated NFE2L2-KEAP1 signalling might cooperate in liver tumourigenesis. As NFE2L2-KEAP1 signalling is known to prevent apoptosis and promote cell survival [42], but constitutive activation of NFE2L2-KEAP1 signalling in *Keap1* knockout mice is not sufficient to drive tumour development [43], it is tempting to speculate that NFE2L2 may not initiate tumourigenesis, but rather confers a high survival capacity and thereby positively selects for already Wnt-activated premalignant cells.

Nevertheless, further genome-wide approaches deciphering genetic and epigenetic alterations on a larger cohort of patients are warranted and will hopefully shed light onto the cause for the widespread activation of the NFE2L2-KEAP1 pathway and a possible crosstalk between Wnt and NFE2L2-KEAP1 signalling in liver cancers.

The establishment of molecular markers to aid risk stratification of cancer patients is an ongoing endeavour in paediatric oncology. Here, we provide evidence that determining the activity of the NFE2L2-KEAP1 pathway might help to identify patients at risk for worse outcome. Accordingly, high *NQO1* expression was associated with two clinical high-risk features, namely metastatic spread and invasive growth into vessels, and consistent with this we found a significantly poorer outcome of high expressing patients. In line with this, the C2 subtype of the 16-gene signature that has been validated to predict poor prognosis in HB [9] was also significantly associated with high *NQO1* expression. Based on these data we advocate to include the measurement of *NQO1* expression into the upcoming international treatment protocol for the pre-therapeutic risk assessment of HB patients in order to aid risk-adapted therapy.

Collectively, our data indicate that activation of Wnt signalling in concert with activation of the NFE2L2-KEAP1 pathway might be the driving force in the development of liver cancers, both in children and adults, thereby offering a new therapeutic target for the treatment of these devastating diseases. Strikingly, a first promising NFE2L2 inhibitor has recently been described, which reduces the protein level of NFE2L2 regardless of the status of *KEAP1* or *NFE2L2* being wild type or mutated [44].

**Financial support**

This work was supported by the German Cancer Consortium (DKTK), the Bettina Bräu foundation (Munich), and the Gänseblümchen foundation (Voerde).

**Conflict of interest**

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

**Authors' contributions**

M. Eichenmüller, F. Trippel, M. Kreuder, and A. Beck designed experiments and acquired, analysed and interpreted data, T. Schwarzmayr performed exome data curation, data filtering and mutation extraction, B. Häberle contributed clinical data and scientific advice, S. Cairo performed statistical analysis, interpreted data and critically reviewed the manuscript, I. Leuschner reviewed the histopathology and provided samples, D. von Schweinitz provided samples, contributed clinical data and critically reviewed the manuscript, T.M. Strom performed exome sequencing and provided computational support, R. Kappler conceived the study, obtained funding, designed experiments, analysed and interpreted data, wrote the manuscript, and directed the overall research. All authors discussed the results and implications and commented on the manuscript.

**Acknowledgements**

We are grateful to Fatemeh Promoli for technical assistance. Dr. Ben Major (University of North Carolina, USA) provided the pKEAP1-Flag expression plasmid, Dr. Masayuki Yamamoto (Tohoku University, Japan) the pNQO1-ARE luciferase reporter, and Dr. Torsten Pietsch (University of Bonn, Germany) the cell lines HepT1 and HepT3.

**Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.08.009.

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## 4.2 Publication II

**Beck A.**, Eberherr C., Hagemann M., Cairo S., Häberle B., Vokuhl C., von Schweinitz D., Kappler R. *Connectivity map identifies HDAC inhibition as a treatment option of high-risk hepatoblastoma.* Cancer biology & therapy 2016; 17:1168-76. (Impact (2016)=3.294)

CANCER BIOLOGY & THERAPY
2016, VOL. 17, NO. 11, 1168–1176
http://dx.doi.org/10.1080/15384047.2016.1235664

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RESEARCH PAPER

# Connectivity map identifies HDAC inhibition as a treatment option of high-risk hepatoblastoma

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**ABSTRACT**

Hepatoblastoma (HB) is the most common liver tumor of childhood, usually occurring in children under the age of 3 y. The prognosis of patients presenting with distant metastasis, vascular invasion and advanced tumor stages remains poor and children that do survive often face severe late effects from the aggressive chemotherapy regimen. To identify potential new therapeutics for high risk HB we used a 1,000-gene expression signature as input for a Connectivity Map (CMap) analysis, which predicted histone deacetylase (HDAC) inhibitors as a promising therapy option. Subsequent expression analysis of primary HB and HB cell lines revealed a general overexpression of *HDAC1* and *HDAC2*, which has been suggested to be predictive for the efficacy of HDAC inhibition. Accordingly, treatment of HB cells with the HDAC inhibitors SAHA and MC1568 resulted in a potent reduction of cell viability, induction of apoptosis, reactivation of epigenetically suppressed tumor suppressor genes, and the reversion of the 16-gene HB classifier toward the more favorable expression signature. Most importantly, the combination of HDAC inhibitors and cisplatin – a major chemotherapeutic agent of HB treatment - revealed a strong synergistic effect, even at significantly reduced doses of cisplatin. Our findings suggest that HDAC inhibitors skew HB cells toward a more favorable prognostic phenotype through changes in gene expression, thus indicating a targeted molecular mechanism that seems to enhance the anti-proliferative effects of conventional chemotherapy. Thus, adding HDAC inhibitors to the treatment regimen of high risk HB could potentially improve outcomes and reduce severe late effects.

**ARTICLE HISTORY**
Received 2 May 2016
Revised 2 August 2016
Accepted 4 September 2016

**KEYWORDS**
Cisplatin; connectivity map; HDAC inhibitor; hepatoblastoma; SAHA; therapy

## Introduction

Hepatoblastoma (HB) is the most common liver tumor of childhood, usually occurring in children under the age of 3 y.[1] Over the last decades tremendous improvement has been made in the stratification and treatment of this highly malignant tumor. Nonetheless, patients that present with metastases, vascular invasion or vast tumor extension are still facing a poor outcome with overall survival rates of only 60%.[2,3] The SIOPEL 4 protocol, which was designed to treat those high risk patients, utilizes a very aggressive regimen of chemotherapy resulting in severe long term side effects in surviving children.[4,5] Those late effects include cardiomyopathy, congestive heart failure and development of second cancers from high doses of doxorubicin as well as permanent hearing impairment and kidney damage from platin derivatives.[6-8] New targeted treatment options hold the potential not only to improve outcome in high risk patients, but to help minimize late effects. Therefore, the identification of potentially druggable targets in HB remains critical.

Besides clinical parameters, gene expression signatures have shown great value in the stratification of HB.[9] A 16-gene HB classifier is able to discriminate between 2 subclasses of tumors and was equally effective in predicting prognosis compared to clinical and histological tumor staging. The C1 subclass is associated with an early tumor stage and a favorable patient outcome, whereas the C2 subclass is tied to advanced tumor stage, metastases, vascular invasion and poor prognosis.[9]

Gene expression profiles that correlate with specific phenotypic features of a disease are also used in the identification of potential new treatment options utilizing biomedical software called the Connectivity Map (CMap).[10,11] CMap is able to link gene expression patterns associated with a distinct phenotype or disease to corresponding patterns derived from drug-treated cancer cell lines. This *in silico* approach has already been successfully used to identify new potential therapeutics for a variety of cancers.[12-14]

Over the last few years it became clear that epigenetic chromatin modifiers, such as histone deacetylases (HDACs),

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Supplemental data for this article can be accessed on the publisher's website.

play a crucial role in the development of various malignancies by aberrantly silencing tumor suppressor genes.[15] HDACs catalyze the removal of acetyl groups from lysine residues on core histones. This leads to a more compact chromatin structure, making it less accessible to specific transcription factors and general transcription machinery as well as altering gene expression toward cancer initiation and progression.[16] HDAC inhibition (HDACi) has shown great promise as a treatment option of tumor entities, in which those epigenetic regulators are overexpressed or deregulated.[16]

By using CMap [10] and publically available gene expression data [9] we identified HDAC inhibitors as one promising molecule class for future therapeutic intervention of high risk HB. We show that HDACs are overexpressed in HB primary tumors and cell lines and that HDACi is able to reduce cell viability and induce apoptosis in HB cells. Furthermore, we demonstrate that HDACi also leads to re-expression of HB-specific tumor suppressor genes and attenuation of the adverse C2 subclass 16-gene expression in HB cells. Finally, we reveal novel therapeutic synergies between cisplatin and HDAC inhibitors, which increase the efficacy of the treatment and lead to a substantial dose reduction of cisplatin. These findings suggest that HDACi is a potential new therapy option for high risk HB.

## Results

### *Connectivity map identifies HDAC inhibitors as potential treatment option of high risk HB*

To identify new treatment options for high risk HB we used the Connectivity Map (CMap), a bioinformatic tool that shows functional connections between drugs and gene expression signatures of diseases.[10] We built an expression signature from existing data derived from 13 primary HB.[9] The signature contained 1,000 genes that best discriminated the high risk-related C2 subtype from the standard risk-related C1 subtype of HB (Suppl. Table 1). C2 tumors within this cluster were associated with poor survival, distant metastasis, vascular invasion, and advanced PRETEXT stages (Fig. 1A). We then used the discriminating signature as an input query for CMap and specifically looked for compounds with negative correlation scores, indicating potential therapeutic value for high risk patients. Out of 1,309 compounds represented by CMap, 2 known inhibitors of PI3K/AKT signaling that have already shown therapeutic effects in HB,[17,18] namely LY-294002 and sirolimus, were highly ranked in the CMap screen (Table 1), thereby underscoring the capability of Cmap in identifying relevant drugs. More interestingly, 2 known HDAC inhibitors were within

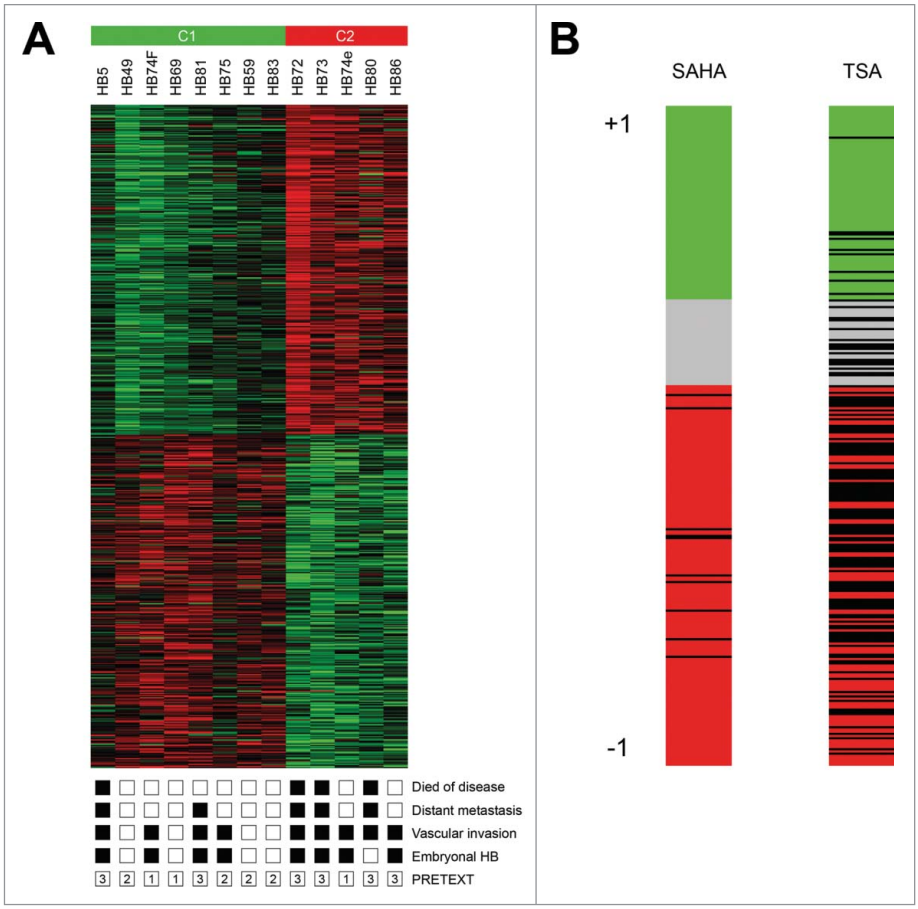


**Figure 1.** (A) Hierarchical clustering of the 1,000 best discriminating genes between the standard risk C1 and the high risk C2 HB subclasses. Important clinicopathological characteristics are depicted below. A detailed list of the genes can be found in Suppl. Table 1. (B) Bar graphs represent the Connectivity Score data for vorinostat (SAHA) and trichostatin A (TSA). The black horizontal lines represent each instance performed with the respective compound. Instances in the red area indicate negative correlation scores and instances in the green area positive ones. No correlation can be detected for instances in the gray area.

**Table 1.** Results of Connectivity Map analysis.

| Rank | CMap name | mean | n | enrichment | *P*-value | Percent |
|---|---|---|---|---|---|---|
| 1 | vorinostat | −0.541 | 12 | −0.475 | 0.005 | 100 |
| 2 | thioridazine | −0.492 | 20 | −0.434 | <0.001 | 90 |
| 3 | prochlorperazine | −0.489 | 16 | −0.374 | 0.017 | 87 |
| 4 | chlorpromazine | −0.474 | 19 | −0.343 | 0.017 | 84 |
| 5 | trifluoperazine | −0.473 | 16 | −0.420 | 0.005 | 75 |
| 6 | α-estradiol | −0.452 | 16 | −0.330 | 0.047 | 81 |
| 7 | trichostatin A | −0.426 | 182 | −0.286 | <0.001 | 85 |
| 8 | fluphenazine | −0.426 | 18 | −0.322 | 0.039 | 83 |
| 9 | tretinoin | −0.396 | 22 | −0.303 | 0.027 | 81 |
| 10 | 15-delta prostaglandin J2 | −0.372 | 15 | −0.401 | 0.011 | 66 |
| 11 | LY-294002 | −0.371 | 61 | −0.260 | <0.001 | 72 |
| 12 | tanespimycin | −0.358 | 62 | −0.212 | 0.007 | 72 |
| 13 | sirolimus | −0.311 | 44 | −0.242 | 0.010 | 70 |

the top matches for the C2 signature (Table 1), namely vorinostat (SAHA) and trichostatin A (TSA). When we plotted the individual correlation scores of the C2 signature for all instances comprising SAHA treatments (12 in CMap) and TSA treatments (182 in CMap), we found them predominantly to be negative (Fig. 1B). This data suggests that HDAC inhibitors can reverse the C2 signature and might therefore constitute a suitable new treatment option of high risk HB.

### HDACs are overexpressed in HB

As high expression levels of HDACs have been suggested as a positive predictor for the efficacy of HDAC inhibition (HDACi) as a treatment option,[19] we determined the expression levels of class I (*HDAC1, HDAC2* and *HDAC3*) and class IIa HDACs (*HDAC4, HDAC5* and *HDAC7*) in 30 primary HB, 5 liver tumor cell lines and 10 non-tumor liver samples. We found that *HDAC1, HDAC2* and *HDAC4* are generally overexpressed in primary HB compared to normal liver expression, with *HDAC1* and *HDAC2* being also overexpressed in HB cell lines (Fig. 2A). Interestingly, we found tumors exhibiting the high risk C2 signature to be significantly correlated with high expression levels of HDAC 1 and 2 (Fig. 2B). These findings suggest that HB exhibit a strong overexpression of several HDACs, especially of class I that are known to be associated with higher tumor grades, aggressive phenotypes and poor prognosis in other solid tumors.[20,21]

### HDACi inhibits growth and induces apoptosis in HB cells

To investigate the effect of HDACi on HB cells, we evaluated the impact of 2 HDAC inhibitors as a monotherapy on the viability of various liver tumor cell lines and fibroblasts as a control, namely SAHA, a pan inhibitor that is able to block the activity of all HDAC subclasses and MC1568, a subclass inhibitor that blocks only class IIa HDACs. Treatment with SAHA resulted in a potent reduction of cell viability in a dose dependent manner (Fig. 3A). Monotherapy of HB cells with MC1568 affected cell viability only at its highest concentration (10$\mu$M). Although expression levels of HDAC4 (class IIa) are high in the primary tumors, they are surprisingly low in the cell lines (Fig. 2A), which might explain the ineffectiveness of class IIa inhibition *in vitro*. Both HDAC inhibitors showed little or no effect on the non-cancerous fibroblasts, suggesting a selective effect of HDACi on liver tumor cells through inhibition of class I HDAC activity.

In order to see how HDAC inhibitors convey their anti-proliferative effects in liver tumor cells we conducted cell cycle and apoptosis analyses. Cells showed only minimal changes in cell cycle progression when treated with either SAHA or MC1568 (Fig. 3B). Interestingly, SAHA treated cells showed a strong induction of apoptosis (Fig. 3B). MC1568 did also induce apoptosis, but to a much lower extent (Fig. 3B). Additional protein analysis unveiled high levels of cleaved PARP after cells underwent HDACi, furthering the assumption that HDAC inhibitors convey their anti-proliferative capabilities rather through induction of apoptosis than cell cycle arrest (Fig. 3C).

Given the fact that the overexpression of HDACs in tumors has been identified as a key factor of aberrant epigenetic tumor suppressor silencing,[22] we examined the expression levels of genes epigenetically silenced in HB [23-25] after treatment with HDAC inhibitors. We found hedgehog-interacting protein (*HHIP*), secreted frizzeled-related protein 1 (*SFRP1*) and insulin-like growth factor-binding protein 3 (*IGFBP3*) to be strongly re-expressed upon HDACi in most cell lines (Fig. 3D), thereby suggesting that the pro-apoptotic effect of HDAC therapy is functionally linked to restored tumor suppressor expression.

### HDACi is able to change the unfavorable gene expression signature in HB cells

The results of the CMap analysis suggest that HDAC inhibitors can potentially reverse the unfavorable C2 expression signature toward the prognostic more favorable C1 signature, virtually turning a high risk HB into a standard risk HB with a better response to therapy and a better outcome. In order to validate the prognostic value of this system for our cohort of 30 primary tumors, we first tested for correlations between HB-subclass (C1/C2) and clinicopathological characteristics. We found that tumors of the C2 subclass were in fact significantly associated with poor survival (Fig. 4A), metastasis, vascular invasion, advanced SIOPEL stage and the unfavorable embryonal histotype (Table 2). As high expression of the genes *RPL10A, E2F5, NLE1, BUB1, DLG7, IGSF1, AFP* and *DUSP9* is characteristic of high risk C2 subclass tumors,[9] we analyzed various liver tumor cell lines that initially show the adverse C2 expression profile after treatment with HDAC inhibitors. In line with the CMap data, we found a strong decrease in expression of most of those genes upon treatment, suggesting a shift from the high risk C2 signature (associated with high expression of the indicated genes) to the standard risk C1 signature (associated with low expression levels of the indicated genes) (Fig. 4B).

### Combination of HDACi and cisplatin show strong synergies

Recent studies suggest that HDAC overexpression is responsible for chemoresistance in solid tumors [19,26] and that HDAC inhibitors can sensitize those tumors to conventional chemotherapy, especially to cisplatin.[27,28] As a proof of concept, we combined each HDAC inhibitor and cisplatin at various concentrations and compared the effects on cell viability with those

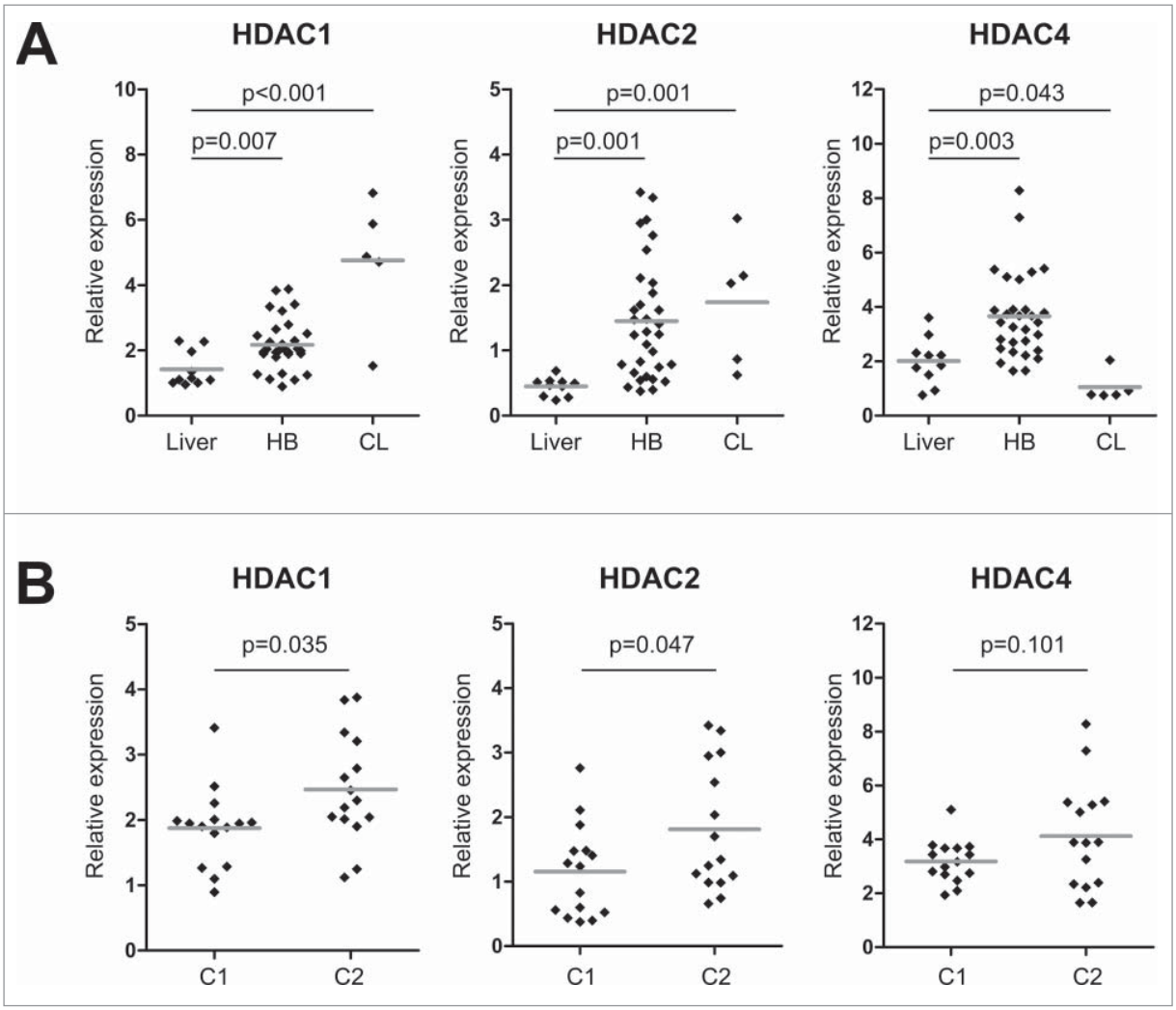


**Figure 2.** (A) HDAC expression levels of normal liver, primary HB and liver tumor cell lines (CL). Expression of class I and class IIa HDACs were measured by qRT-PCR and normalized to the expression of the house-keeping gene *TBP*. Statistical significance was calculated for differences between normal liver tissue and tumors and tumor cell lines using t-test. (B) HDAC expression levels of primary HB after their stratification as standard (C1) or high risk HB (C2) according to the 16-gene HB classifier. Statistical significance was calculated for differences between C1 and C2 tumors using t-test.

of a combination of doxorubicin and cisplatin, which constitutes the SIOPEL4 regimen.[5] When we analyzed the viability data of the combinational therapy and compared them to the monotherapy data of each individual compound by using the CompuSyn software, which is able to identify synergies between 2 compounds, we found synergistic effects between the HDAC inhibitors and cisplatin in all cell lines and at most concentrations (Fig. 5A). Notably, only few synergies were detected between doxorubicin and cisplatin.

When we looked at cell viability we found that combinations of cisplatin and SAHA were equally effective compared to combinations of cisplatin and doxorubicin (data not shown). We found the strongest synergies between 5$\mu$M cisplatin and various concentrations of SAHA. Combinations at this particular concentration appear to be slightly superior to the combination of cisplatin and doxorubicin at equal concentrations in regard to cell viability (Fig. 5B). While MC1568 and cisplatin combinations also showed a considerable effect on cell viability, it did not achieve the effect of cisplatin and doxorubicin combinations at any concentration.

## Discussion

Since standard risk HB patients can achieve good outcomes with already existing therapy regimens,[2] we focused our research on the discovery of new treatment options for high risk patients, whose outcome still remains poor with the current treatment strategies.[3,5] Using CMap, we identified HDAC inhibitors as potential new drugs for the treatment of high risk HB. Interestingly, previous studies have already shown antiproliferative effects of HDACi *in vitro* [29-31] and in preclinical mouse models of hepatocellular carcinoma (HCC),[32] the most common liver tumor in adults with a considerably poor outcome. Consequently, the pan HDAC inhibitor belinostat has recently been tested in a clinical phase I/II trial for patients with unresectable HCC, which resulted in disease stabilization with a tolerable toxicity profile.[33] Moreover, as phase I clinical trials have already demonstrated the safe use of SAHA in pediatric populations,[34,35] based on earlier preclinical *in vitro* studies using SAHA with doses similar to the ones in our experiments,[36-38] it could be emphasized that these doses are

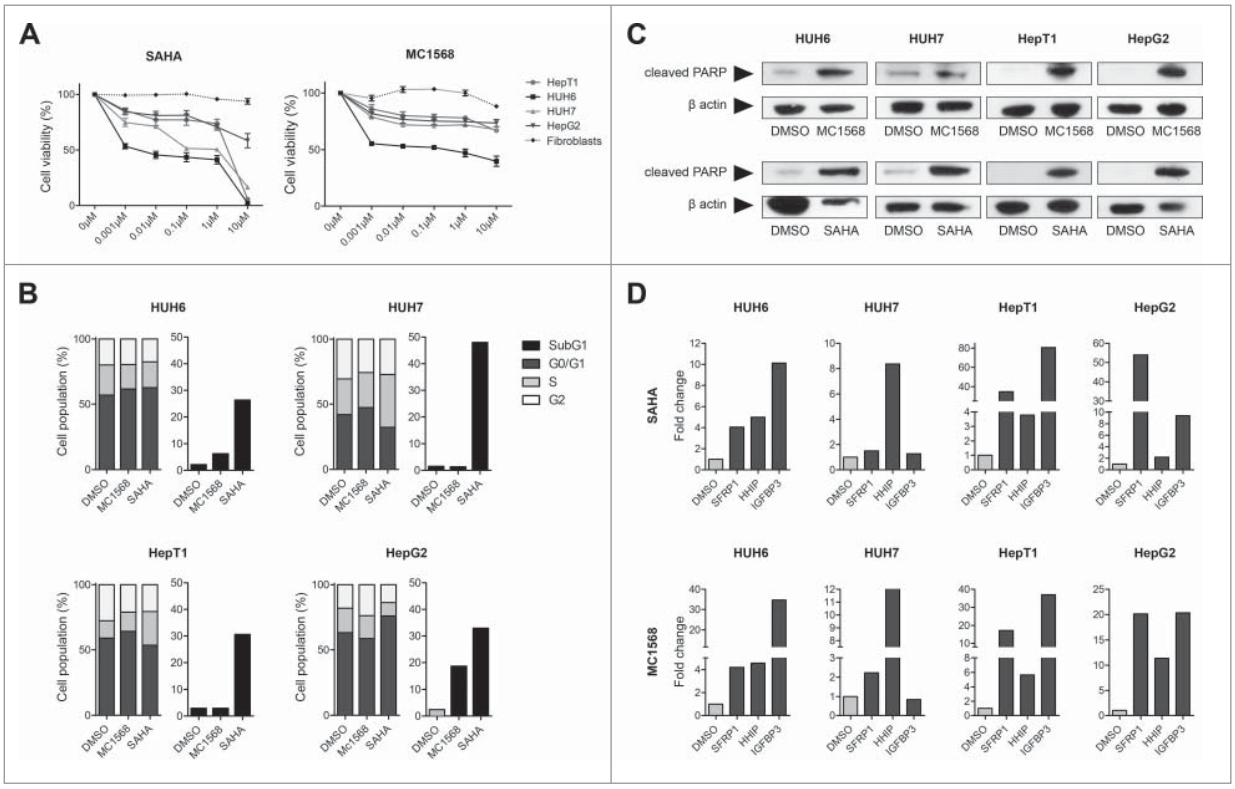


**Figure 3.** (A) Cell viability of HB cell lines and fibroblasts as evaluated by MTT assay after 48 h treatment with indicated concentrations of MC1568 and SAHA. Values represent means ± standard deviation of 3 independent experiments performed in duplicates. (B) Cell cycle distribution (left panel) and apoptosis (right panel) of liver tumor cell lines were analyzed by flow cytometry 48 h after treatment with vehicle (DMSO), 10$\mu$M of MC1568 and 1$\mu$M (HUH6, HepT1 and HepG2) or 2$\mu$M of SAHA (HUH7). (C) Western blot analysis for cleaved PARP in indicated HB cell lines after 48 h of treatment with DMSO, MC1568 and SAHA (concentrations as in B). (D) Expression levels of the tumor suppressor genes *SFRP1*, *HHIP*, and *IGFBP3* in liver tumor cell lines after HDACi. Expression levels were measured after 48 h of treatment by qRT-PCR and normalized to the expression of the house-keeping gene *TBP*. Indicated are fold changes to the individual DMSO control.

clinically achievable and tolerable. Thus, our findings are in line with studies in HCC and support the concept of HDACi as a promising treatment strategy for liver malignancies, both in the adult and pediatric population.

Our systematic expression analysis of a large set of primary HB and liver tumor cell lines revealed that HDACs are generally upregulated compared to normal liver tissue. Overexpression of *HDAC2* has been suggested as a positive predictive marker for the response of solid tumors to treatment with HDAC inhibitors.[19,39,40] Treatment of liver tumor cells with HDAC inhibitors seem to support this theory, given the fact that the cell lines with high *HDAC2* expression (HUH6 and HUH7) were most responsive to HDACi, whereas cell lines with normal *HDAC2* expression (HepG2 and HepT1) responded poorly to the monotherapy regimen. However,

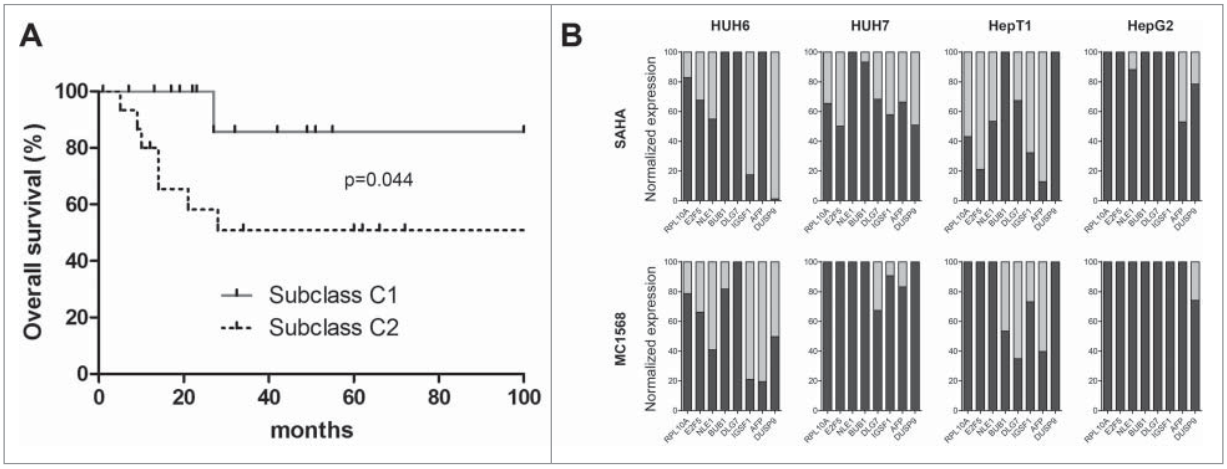


**Figure 4.** (A) Overall survival was calculated as time from diagnosis to death of the disease and is plotted for 30 HB patients. Statistical significance was calculated using the Mantel-Cox test. (B) Cell lines expressing the adverse C2 signature were treated with 10$\mu$M of MC1568, 1$\mu$M (HUH6, HepT1 and HepG2) and 2$\mu$M (HUH7) of SAHA, or vehicle (DMSO). Graphs show decreased expression levels in percent of 8 high risk C2 signature genes of the indicated genes upon 48 h of treatment with the indicated HDAC inhibitors.

**Table 2.** Association between C1/C2 classification and clinicopathological features of HB.

| Factors | No. of tumors | C1 | C2 | *P*-value |
|---|---|---|---|---|
| Sex | | | | 0.715 |
| Male | 15 | 7 | 8 | |
| Female | 15 | 8 | 7 | |
| Age at diagnosis (months) | | | | 0.232 |
| <36 | 21 | 12 | 9 | |
| >36 | 9 | 3 | 6 | |
| Histological type | | | | 0.003 |
| Fetal | 23 | 15 | 8 | |
| Embryonal | 7 | 0 | 7 | |
| Stage (PRETEXT) | | | | 0.068 |
| 1–3 | 24 | 14 | 10 | |
| 4 | 6 | 1 | 5 | |
| SIOPEL risk group | | | | 0.001 |
| Standard risk | 15 | 12 | 3 | |
| High risk | 15 | 3 | 12 | |
| Outcome | | | | 0.013 |
| Alive | 22 | 14 | 8 | |
| Dead | 8 | 1 | 7 | |
| Metastasis | | | | 0.003 |
| No | 16 | 12 | 4 | |
| Yes | 14 | 3 | 11 | |
| Vascular invasion | | | | 0.031 |
| No | 23 | 14 | 9 | |
| Yes | 7 | 1 | 6 | |
| Multifocality | | | | 0.409 |
| No | 22 | 12 | 10 | |
| Yes | 8 | 3 | 5 | |

if *HDAC2* has the potential to be used as a biomarker for predicting clinical responses to HDACIs, as shown for HR23B in adult HCC,[33] has to be confirmed in future studies.

The changes induced by HDACi in the gene expression of HB cells that initially showed the adverse C2 signature not only underlines the predictive power of the CMap by partially reversing this signature, but also suggests a specific benefit of HDACi for patients with high-risk HB. Since our analysis showed a strong association between the C2 signature of primary tumors and clinicopathological features, such as poor survival, metastasis, vascular invasion and advanced tumor stage, a HDACi induced change in gene expression toward the more favorable C1 signature could potentially lead to a better outcome and an enhanced response to treatment.

Late effects of chemotherapy constitute an increasing problem, given the tremendous progress that has been made in achieving better long-term survival rates for children with cancer. Doxorubicin, the chemotherapeutic currently used to escalate the therapy of high-risk HB, is among the agents with the most severe late effects such as cardiomyopathy, congestive heart failure and development of secondary malignancies.[4,8] Our data suggest that doxorubicin can possibly be replaced with the HDAC inhibitor SAHA as the escalating agent for the high risk HB group in addition to the cisplatin backbone, without compromising the efficacy of the treatment. Furthermore, HDAC inhibitors, especially SAHA, have shown great promise in overcoming chemoresistance in solid tumors. Studies provide evidence that HDACi can sensitize tumor cells for chemotherapeutics, especially cisplatin.[26,27] The strong synergies we found between SAHA and cisplatin hold the potential to reduce cisplatin concentrations, which would further diminish the late effects caused by this agent, namely permanent hearing impairment and kidney damage.[4,6,7]

While further studies are warranted to reveal whether HDACi also represents a successful treatment option *in vivo*, which could be preclinically tested in genetic or patient-derived xenograft mouse models, our data suggest a direct benefit of HDACi for children with high-risk HB through a more targeted approach. This could potentially open up new therapeutic opportunities for future clinical studies in which substituting conventional chemotherapeutic agents with HDACi could reduce detrimental long-term side effects in patients.

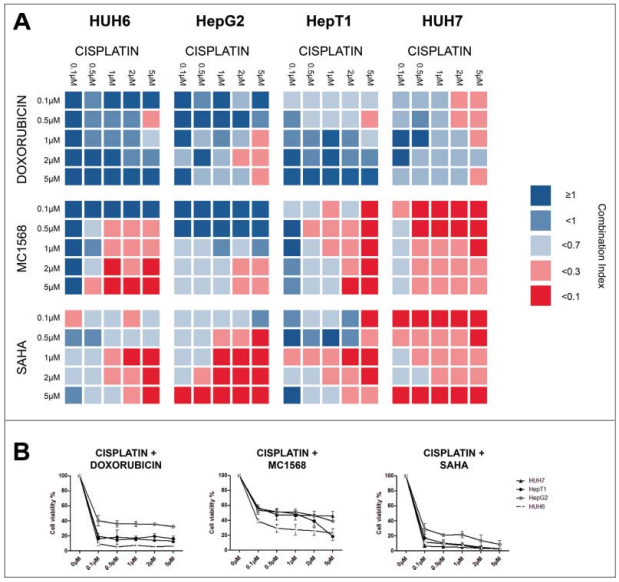


**Figure 5.** (A) Cell lines were treated with the indicated concentrations of cisplatin combined with various concentration of either doxorubicin, MC1568 or SAHA. Cell viability was measured after 48 h and combination indices (CI) were calculated from 2 independent experiments performed in duplets. CI < 0.1 = very strong synergism, CI < 0.3 = strong synergism, CI < 0.7 = synergism, CI <1 = slight synergism, CI ≥ 1 no synergism. (B) Cell lines were treated with the indicated concentrations of cisplatin combined with 5$\mu$M of either doxorubicin, MC1568 or SAHA. Cell viability was measured after 48 h from 2 independent experiments performed in duplets.

## Patients and methods

### *Connectivity map analysis*

Expression profiling data from 13 primary HB with defined histological and clinical annotations (Suppl. Table 3) were obtained from ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) under the accession numbers E-MEXP-1851.[9] After using this data to identify the most differentially expressed genes between previously defined standard risk tumors (C1) and high risk tumors (C2), we built a gene expression profile containing the 1,000 best discriminating genes between these 2 subclasses (Suppl. Table 1). We entered this signature into the latest dataset of CMap (Build 02) and compared it to more than 7,000 so-called instances, which are defined by expression profiles of human cancer cell lines treated with 1,309 therapeutic compounds at different concentrations (http://www.broadinstitute.org/cmap). Each instance was assigned a connectivity score from −1 to 1, representing the relative association of the respective instance with the specific query. A positive connectivity score indicates that a drug is able to induce the input

signature in human cell lines. Conversely, a negative connectivity score indicates that a drug is able to reverse the input signature. Since we used the high risk C2 signature as input, we looked for negative connectivity scores, which indicate potential therapeutic value. After rank-ordering all instances, the connectivity score of various instances of the same compound were averaged and filtered by the number of instances (n >10) and *P*-value (<0 .05).

### Patients and materials

A total of 30 liver tumor specimens were obtained from pediatric patients undergoing surgical resection in our department. Matching normal liver was available from 10 patients. Written informed consent was obtained from each patient, and the study protocol was approved by the Committee of Ethics of the Ludwig-Maximilians-University of Munich. We used the 4 human HB cell lines HepT1, HepT3 (both provided by Dr. T. Pietsch), HepG2 (ATCC, Manassas, VA, USA), and HUH6 (Japanese Collection of Research Bioresources, Osaka, Japan), the hepatocellular carcinoma cell line HUH7 (kindly provided by Dr. Enrico de Toni), as well as human fibroblasts, which were obtained from a skin biopsy of a healthy male volunteer. Cells were grown at 37°C in RPMI medium containing 10% FCS, 1% antibiotics and glutamine supplement.

### Real-time reverse transcription polymerase chain reaction

RNA extraction and purification, cDNA synthesis, PCR amplifications and quantization of gene expression were performed as described before [25] using the primer pairs outlined in Suppl. Table 2. Amplification of the house-keeping gene TATA-Box-binding-Protein (*TBP*) was performed to standardize the amount of sample RNA.

### Proliferation assays and detection of synergy

Cell proliferation was assessed using 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assays. Cells were seeded at a density of 10,000 cells per well into 96 well plates (NUNC, Langenselbold, Germany). After overnight attachment, cells were treated for 48 hours with various concentrations of suberoylanilide hydroxamic acid (SAHA, Sigma-Aldrich, Steinheim, Germany), MC1568 (Selleck Chemicals, Munich, Germany), cisplatin (Selleck Chemicals), doxorubicin (Selleck Chemicals), DMSO (Sigma-Aldrich) or various combinations of those compounds. To assess cell viability, the optical density was measured at a wavelength of 595 nm after the addition of MTT using the GENios multi scanner microplate reader (TECAN, Männedorf, Switzerland). Synergy testing of the combined treatment was analyzed using the CompuSyn software (http://www.combosyn.com/), which utilizes the Chou-Talalay method.[41] The calculated combination index (CI) was used as a quantitative measure of the degree of interaction between 2 drugs. CI = 1 indicates additivity, CI > 1 indicates antagonism, and CI < 1 indicates synergism.

### Apoptosis and cell cycle analysis

Cells were seeded in 6 well plates and after 24 hours, exposed to DMSO, SAHA or MC1568 at various concentrations for 48 hours. Fixation and permeabilization of cells were performed by dropwise addition of 70% ethanol while vortexing and incubation at −20°C for at least 2 hours. Permeabilized cells were washed with PBS and DNA was stained using 0.02 mg/ml propidium iodide (Sigma-Aldrich) and 0.2 mg/ml RNaseA (Qiagen, Hilden, Germany) in PBS/0.1%Triton X-100 (Sigma-Aldrich) for 30 minutes at room temperature in the dark. Cell cycle was analyzed via BD-LSRFORTESSA flow cytometer (BD Biosciences, San Jose, CA, USA) and using Flowing software 2.5.1 (http://www.flowingsoftware.com/).

### Western blot

Cells were seeded at a density of $1 \times 10^6$ per 10 cm cell culture dish. After overnight attachment cells were treated for 48 hours with SAHA, MC1568 or DMSO at various concentrations. After treatment non-adherent cells and adherent cells were pooled together in ice-cold lysis buffer (0.5% Triton-X100, 1 mM orthosodiumvanadate, cOmplete Mini protease inhibitor (Roche Diagnostics, Penzberg, Germany)). Protein lysates were incubated on ice for 20 minutes under occasional vortexing. After centrifugation for 30 minutes at 4°C protein lysates without cell debries were stored at 4°C until use. The protein concentration was determined by the Bio-Rad Protein Assay (München, Germany). Proteins (20 $\mu$g) were loaded on a 4–12% BIS TRIS NuPage Gel (Novex by Life Technologies, Carlsbad, CA, USA) separated under reducing conditions and transferred to nitrocellulose blotting membrane (GE Healthcare Life Sciences, Freiburg, Germany). Thereafter, membranes were blocked with PBS/0.1% Tween20 and 5% non-fat dry milk for 2 hours at room temperature. First, antibodies rabbit anti-human poly(ADP-ribose) polymerase (PARP) (1:1,000) or rabbit anti-human $\beta$-actin (1:2,500) (all from Cell Signaling Technology, Leiden, Netherlands) were added over night at 4°C. For detection, membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated polyclonal goat anti-rabbit immunoglobulin secondary antibody (Dako, Glostrup, Denmark) and signals were captured using the enhanced Western blotting reagent detection system (GE Healtcare, Buckinghamshire, UK).

### Statistical analyses

Data were expressed as means + standard deviation (SD) and statistically subjected to Student's unpaired *t*-test. Kaplan-Meier estimates of specific survival time in the various groups were compared using the log-rank Mantel-Cox test. A level of $P < 0.05$ was considered to be significant, $P < 0.01$ highly significant.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

## Funding

This work was supported by grants of the Bettina Bräu foundation, Munich, Germany and the Gänseblümchen-Voerde foundation, Voerde, Germany (to R.K.). We are grateful to Fatemeh Promoli for technical assistance.

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# 6. Acknowledgement

I want to thank Prof. Dr. med. Dietrich von Schweinitz, Director of the Department of Pediatric Surgery at the Dr. von Hauner Children's Hospital in Munich for giving me the opportunity to conduct my research at his institution.

My deepest gratitude goes to Prof. Dr. rer. nat. Roland Kappler who has become a great mentor to me and was nothing but helpful during my time in his laboratory. His guidance is what made me a decent scientist and I am especially grateful for his trust in my research and the opportunity to publish my work with his help.

I would also like to thank Fatemeh Promoli and Tatiana Schmid for the help they provided to my research and for everything they taught me. The same applies to all my other colleagues that I had the privilege of working with over the past few years.

Finally I would like to thank my family, especially my wife Felicitas for her endless patience and encouragement.